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EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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BLOOD CALCIUM DISTRIBUTION IN ANAPHYLAXIS IN THE GUINEA PIG.

By HERMAN BROWN and SUSAN GRIFFITH RAMSDELL.

(*From the Research Institute of Cutaneous Medicine, Philadelphia.*)

(Received for publication, January 24, 1929.)

The biological importance of calcium has, of course, long been known; but it has only been comparatively recently, through the stimulus afforded by the findings of the close relationship between certain pathological conditions and disturbed calcium metabolism, that the element has been so extensively studied. The effect of calcium on tissue permeability and irritability, and the rôle attributed to it in blood coagulation is of special interest in the study of anaphylaxis; and the work to be reported here is a study of the variations in the total calcium and in the diffusible and non-diffusible fractions which occur in the anaphylactic guinea pig.

While many determinations have been made of total serum calcium, usually with findings of a constant or only slightly diminished value (1–3), a review of the literature yields no report on diffusible calcium in anaphylactic shock. Blum, Delaville and Caulaert (4) found the diffusible calcium in peptone shock in one dog increased from 5.7 to 7.5 mg. per 100 cc.; and in the rabbit, in which the manifestations of shock were weak, two experiments gave nearly double the values for the diffusible fraction over that found in the control animal. No such study is to be found for the guinea pig, in which, of all the experimental animals, anaphylactic shock is to be demonstrated most uniformly and definitely.

Technic.

For this study 66 guinea pigs were used, including the normal controls. The shocked animals were prepared by direct sensitization with human or horse serum, or passively by the transfer (48 hours) with an anti-horse serum (rabbit). Since the size of the animal precluded preshock bleedings, subsensitized animals, for controls, were injected with horse serum and then killed after varying intervals corresponding to those at which the shocked animals were bled.

In all the groups it was necessary to use the pooled bloods of from 2 to 4 animals, depending upon their size, in order to secure a sufficient sample of serum for analysis. Care was taken, in all cases, that the animals in each group showed the same type of reaction, and that the bleedings were made at the same intervals after injections.

Calcium determinations were made according to Tisdall's modification (5) of Kramer and Tisdall's method (6). The method of Moritz (7) as modified by Updegraff, Greenberg and Clark (8) was used in obtaining the diffusible calcium findings. As a criterion of a proper permeability of the collodion bags, we accepted only those results in which not less than 1 cc. of fluid passed through the membrane and which, of course, gave no tests (biuret) for protein in the diffusate. Nearly all results given in the table are averages of two determinations and all the results were actual ones, *i.e.*, the non-diffusible calcium was not calculated by difference. This we believe to be an added check on the results, for though the possibility has been suggested (8) that some Ca may be lost in the membrane itself, an inspection of the tables will indicate that the sum of the diffusible and non-diffusible calcium is, in general, rather more, instead of less, than the total calcium, indicating that such losses are quite negligible and are more than counterbalanced by the errors inherent in the calcium determination itself.

DISCUSSION.

In the table are listed the results of determinations for total serum calcium, the diffusible and non-diffusible, and the percentage of these of the total calcium. All the figures are placed in one table to facilitate comparison of the normal findings with those for the animals which were subsensitized and did not show shock symptoms and those which showed a marked degree of anaphylaxis.

An inspection of the table indicates, in the first place, that the total serum calcium is quite constant in the guinea pig even in cases of severe shock. However, much work has been done in recent years to show that the biological activity of calcium really depends upon the various forms in which it may exist. Since the discovery of Rona and Takahashi (9) that serum calcium exists in diffusible and non-diffusible form, much evidence has accumulated (10–12) to indicate that the non-diffusible fraction is in the form of a protein compound. This explanation has been rejected by Cameron and Moorhouse (13) and, though it is not the purpose of this paper to discuss the merits of these theories, we should like to point out, that, at present, it appears to be accepted that the non-diffusible serum calcium is in the form of a protein complex. Moreover, the total serum calcium determination

may be a poor index of what is really happening, for a disturbance of this calcium-protein complex may occur with resulting marked

TABLE I.

Blood Calcium Distribution in the Normal and Shocked Guinea Pig.

Experiment No.	Total Ca	Diffusible Ca		Non-diffusible Ca		Remarks
		Mg. per 100 cc.	Per cent of total	Mg. per 100 cc.	Per cent of total	
	mg. per 100 cc.					
1	10.2	5.4	53	4.5	44	Normal animals
2	8.6	4.6	54	4.0	47	Normal animals
3	10.2	5.9	58	4.0	44	Normal animals
4	8.6	4.7	55	3.6	42	Normal animals
5	9.6	6.5	67	3.2	33	Normal animals
6	10.1	5.3	52	5.0	49	Doubtful shock 20 min. after antigen injection
7	9.4	5.0	53	4.2	45	Negative shock. Bled 20 min. after injection
8	9.4	5.1	54	4.2	45	Doubtful shock. Bled 20 min. after injection
9	8.8	5.2	59	3.5	40	Negative shock. Passive sensitization
10	8.4	4.9	58	3.6	43	Maximum shock; bled at prostration
11	10.0	7.1	71	3.2	32	Maximum shock; bled at prostration
12	9.3	5.9	63	3.2	34	Maximum shock; bled at prostration
13	9.2	6.6	72	2.7	30	Maximum shock; bled at prostration
14	9.7	6.2	64	3.6	37	Maximum shock; bled at prostration; passive sensitization
15	9.3	6.7	72	3.1	33	Immediate shock; bled on appearance of clonic seizures; lungs maximum
16	9.6	5.7	59	4.1	43	Immediate shock; bled on appearance of clonic seizures; lungs maximum
17	10.1	7.3	72	3.0	30	Immediate shock; bled on appearance of clonic seizures; lungs maximum
18	10.0	7.3	73	2.7	27	Histamine, 1 cc. 1/5000 dilution; immediate violent shock; bled at prostration

physiological effects, but without altering the total serum calcium. A striking example of this has recently been pointed out by Shelling and

Maslow (14) who demonstrated that, following citrate additions to serum, either *in vitro* or *in vivo*, practically the entire calcium-protein combination is destroyed, all the calcium becoming diffusible, without, however, altering the total serum calcium. In order to determine if the reaction in the guinea pig following citrate injection resembled anaphylaxis, Shelling and Maslow's procedure was followed in one set of animals: calcium determinations (not listed in the tables) indicated that over 80 per cent of the guinea pig serum calcium became diffusible. Following the citrate injections violent immediate reactions were elicited; these, however, did not in any way simulate those characteristic of anaphylactic shock.

The findings for diffusible calcium, in terms of percentage of total calcium, are shown in the table to vary from 53 to 67 with an average of 55 for the normal animals; for the subsensitized which did not manifest typical symptoms upon reinfection, the variations were from 52 to 59 with an average of 54.5; while in the shocked animals a minimum value of 58 and a maximum of 72, with an average of 66, were found. Experiments 10 and 16 of the shocked animals gave values which may be considered "high" normal, while the results of the other experiments indicated a very definite increase in diffusible calcium with a corresponding decrease in the non-diffusible or "protein-complex" calcium.

There is then no change in this form of calcium incident to the treatment of the animal with a foreign serum, but a decided increase when the animals manifest shock. This increase is apparently not affected by the time interval at which the animal is bled, once symptoms of acute shock are established.

That the calcium change is not peculiar to anaphylactic shock is evidenced by the experiment with citrate injections and with histamine, resulting in the highest figure found for diffusible calcium. But in this last type of reaction, the coagulability of the blood is known not to be affected. This change in calcium ion content of the blood in anaphylactic shock is then not of necessity directly related to the phenomenon of a lessened coagulation time. This last has, indeed, been adequately accounted for by experiments of Weil (15) which demonstrated that the lessened coagulation time could be attributed to a liver product thrown into circulation following contact with the

antigen. From the results found for the non-diffusible fraction, it may be inferred that some change occurs in the protein of the serum whereby its normal combination with calcium is disturbed; but the data at hand furnish no basis for any conclusions as to the rôle this change plays in the basic mechanism of the phenomenon of shock.

CONCLUSION.

The results above reported for total calcium and the membrane-diffusible fraction in the serum of the guinea pig, taken at various intervals during anaphylactic shock, confirm the findings of previous workers that the total calcium is essentially unchanged. There is, however, the further finding that the diffusible fraction is considerably increased over that found for the animal similarly treated but not manifesting characteristic symptoms.

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OXIDATION-REDUCTION EQUILIBRIA IN BIOLOGICAL SYSTEMS.

II. Potentials of Aerobic Cultures of B. typhosus.

By CALVIN B. COULTER, M.D., and MOSES L. ISAACS, Ph.D.

(*From the Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York.*)

(Received for publication, February 2, 1929.)

Gillespie (1) was the first to observe that an indifferent metallic electrode placed in a culture of bacteria manifests a negative drift in potential. Potter (2) had found previously that an electrode in an inoculated portion of culture medium is negative to an electrode in an uninoculated portion of the same medium, when the two portions are separated by a porous diaphragm. Clark (3) and his collaborators have extended the observations of Gillespie and have aroused a widespread interest in the biochemical processes involved in the establishment of reduction potentials.

The hopes of the earlier investigators that the determination of reduction potentials would provide a measure of the intensity of oxidation or reduction in biological systems cannot yet be realized, since difficulties are apparent in the interpretation of such potentials in strict accordance with the theory of oxidation-reduction equilibrium as it has been developed for inorganic or relatively simple organic substances. The substances responsible for the observed potentials of biological complexes have not been identified; there is much to suggest, however, that the sulfhydryl bodies are involved. The work of Dixon (5) and Dixon and Quastel (4) and the recent careful study of Michaelis and Flexner (6) indicate that these bodies do not constitute a simple reversible oxidation-reduction system, as would be required for strict application of the theory of reduction potentials, although their solutions in the absence of oxygen yield definite potentials which vary with the C_H and the concentration of the reduced form of the substance. These substances are, therefore, electromotively active and the combined form glutathione is so generally present in biological preparations that it must be concerned in the production of electrode potentials. These and other electromotively active bodies may also actually intermediate the reduction potentials, acting as catalysts for the oxidation or reduction of organic constituents which are not themselves electromotively active. There is, however, at the present time no evidence that the reactions which may be catalyzed by such substances are subject to definition in terms of intensity values. Further data from biological systems

must be accumulated before a formulation of the mechanisms involved can be attempted.

No satisfactory explanation has been offered for the part played by molecular oxygen in the development of biological reduction potentials, although all investigators have noted that reduction potentials of considerable intensity are attained only when oxygen is removed from the system. This may be accomplished by deaeration by a stream of nitrogen or by a spontaneous oxygen consumption which may take place as in sterile bouillon (7) even when the system contains only inanimate materials. Before such anaerobic conditions are attained the potentials in any biological system are distinctly more positive than when oxygen is absent, and it is not possible to decide to what extent the electrode under these conditions acts simply as an oxygen electrode or measures the actual oxidation-reduction forces which prevail in the system or indeed to decide whether or not these two possible mechanisms are properly to be contrasted.

Limitations thus surround the investigation of biological potentials and the significance which may be attached to the results.

In the first paper (7) of this series the reduction potentials of sterile culture bouillon were examined, and it was found that sterile bouillon in the absence of oxygen yields rather definite and characteristic potential values. Although the significance of these values is limited it seemed possible that they might serve as comparison values in a study of the potentials of growing cultures. The subject of the present paper is the relation of the potentials of sterile bouillon to those of living cultures of *B. typhosus* in the same medium. It was the hope that such an investigation would throw light upon the reducing activities of bacteria and upon the mechanism by which oxidation-reduction potentials are produced.

Technic.

The arrangement of the electrode cell and the method of potential measurement was that described in the first paper of this series (7). The electrodes used were for the most part purified gold which had been employed in the earlier work.[1] An

[1] The electrodes were placed when not in use in chromic acid solution, and were immersed for several minutes in concentrated sulfuric acid before each experiment. It was found that if this precaution were not taken the electrodes were sluggish and did not agree with one another closely. It was believed that the concentrated acid removed traces of stop-cock lubricant which may have been carried into the cell and become attached to the electrodes, but it is possible that solution of a film of oxide on the surface of the electrodes may have been involved. See Michaelis and Flexner (6).

improvement was made in the method of setting up the agar bridge: instead of filling the tube with agar, sterilizing separately, and fitting it aseptically to the large stopper of the electrode cell, the tube was inserted in the stopper and its outer end connected by rubber tubing provided with a pinch-cock to a funnel containing the KCl agar. After sterilization of the completely assembled apparatus in the autoclave at 10 pounds for 10 minutes, the melted agar was allowed to flow, by opening the pinch-cock, into the bridge tube, while the apparatus was still warm. A drop of agar was allowed to form at the tip of the tube, at which time the pinch-cock was closed. After the agar had solidified the connection to the funnel was removed and replaced by a rubber tube, filled with saturated KCl, which was connected to a special form of calomel half-cell through a three-way stop-cock. During an experiment this cock was kept closed and the rubber tube was shellacked, so that the bridge was a closed system into which oxygen could not readily diffuse. Reliance was placed on careful shellacking of all exposed rubber surfaces for exclusion of oxygen which would otherwise diffuse through the rubber.

The electrode cell and its contents were maintained at 38°C. by means of a small heating coil which fitted closely about the cell. A thermometer was placed in and sterilized with the cell. The flow of electric current through the heating coil was controlled by sliding-contact rheostats; a low-range ammeter in the circuit permitted calibration of the current required to reach and maintain any desired temperature. Although a thermostat was not used the temperature was kept within the limits of ± 1.0°C. variation by adjustment of the rheostats.

The bouillon used was standard meat infusion, adjusted to pH 7.6 and buffered by phosphate which was added to a concentration of M/15. A saturated solution of dextrose was sterilized by heating to 80°C. for 30 minutes; an amount of this sufficient to give a final concentration of 0.5 per cent was added aseptically, in those experiments in which a dextrose medium was used, after the bouillon had been autoclaved. For each experiment 25 cc. of bouillon were autoclaved in a small flask, at 10 pounds for 10 minutes, along with the electrode cell. After cooling to about 38°C. the bouillon was transferred aseptically to the separatory funnel of the cell assembly. While in the funnel the bouillon was inoculated with two loops of an 18 hour broth culture of *B. typhosus* and was then allowed to run into the electrode cell. At intervals during many of the experiments portions of culture were drawn off from the cell by means of the bottom outlet, for the purposes of measurement of pH and of enumeration of bacteria by dilution plate cultures.

The electrical measurements were made by means of the usual potentiometer assembly, and at the same time were followed in many of the experiments by a Leeds and Northrup recording potentiometer. This instrument was specially adapted for this work by the makers; the moving coil has a resistance of 2500 ohms and is delicately balanced. This instrument was used chiefly as a precaution against overlooking changes in potential during the hours when the electrode cell was not under actual observation. The potentials indicated by the automatic

recorder were in general within 5 to 10 millivolts of those determined manually. The numerical values referred to in connection with the description of the experiments represent independent readings taken with the more sensitive hand-operated potentiometer.

Measurement of oxidation-reduction potentials was made first on cultures in bouillon which contained only the small amount of dextrose naturally present. In such cultures the change in pH after 24 hours

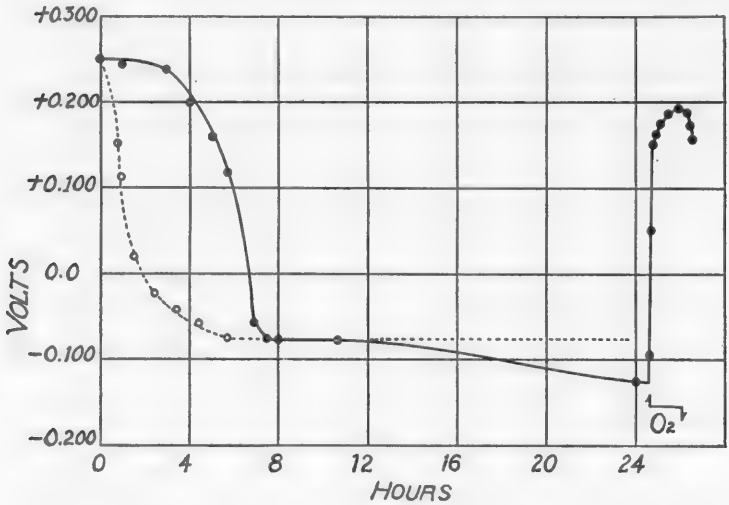

FIG. 1. The potentials of *B. typhosus* in bouillon without added dextrose are given by the solid line. The broken line gives the potentials of the bouillon in the sterile condition when deaerated with nitrogen. The rise in potential of the culture on passage of oxygen and the return to negative values on stopping the flow of oxygen are shown at the right of the figure. The culture throughout incubation had access to atmospheric oxygen. Ordinates represent *Eh* values at pH 7.6.

growth is small, amounting to less than 0.3 pH; if 0.1 per cent or more of dextrose is present the acid resulting from fermentation of the sugar is sufficient to displace the pH of the medium in spite of the addition of phosphate buffer, and introduces a complication in the interpretation of the potentials observed. When the electrode vessel was half filled with bouillon, and the cocks of the gas tubes entering and leaving the cell were closed, the culture had access to an equal volume of air, and

the conditions were similar to those of the ordinary aerobic culture. The time-potential curves of cultures under these conditions have been obtained in a number of experiments.

The course of the potentials is shown for a typical experiment in Fig. 1. During the first few hours the potentials remain close to the initial values, which range as in sterile bouillon at the beginning of deaeration between +0.250 and +0.150 volt. The lower initial values have been given by bouillon which had been freshly autoclaved and rapidly cooled before transference to the cell. This is in agreement with the observation of Dubos (8) on the reducing power of sterile media for dyes. A positive drift in potential from a few millivolts to 0.040 volt has usually been observed beginning shortly after measurements were started, and lasting for 2 to 3 hours; it may be related to the equilibration of the freshly autoclaved bouillon with the air. Within 3 to 6 hours after inoculation there has been observed regularly a gradual decline in potential followed shortly by an abrupt drop to −0.040 to −0.070 volt, and a slower fall to −0.080 to −0.090 volt. This level is maintained for several hours and appears to represent an equilibrium value for the culture during its youth. It was not possible, as will be described later, to make frequent samplings of the culture because of the change in potential which these involved, but it is evident from the form of the growth curve in these and in other observations that the culture has passed through its lag period and is in the period of logarithmic increase in numbers of bacilli during the time when this level of potential is maintained. The potentials then undergo a further gradual decline and after 24 hours have been found very close to −0.125 volt. In one experiment in which observations were made over a longer period of incubation this decline continued and at the end of 53 hours the potential reached the value of −0.145 volt.

The time-potential curve of an aerobic culture during the first 24 hours of growth may thus be divided into 4 portions: (1) the potentials are close and slightly positive to the initial values; (2) a decline, in part abrupt, to −0.080 or −0.090 volt; (3) a period in which the potentials are maintained at this level; (4) a gradual decline to more negative values continuing over a period of several days.

It seems highly probable that the presence of oxygen in the culture

is of primary importance in the establishment of the potentials, and the following explanation has been evolved. In the first portion of the time-potential curve, the small numbers of bacteria introduced as inoculum are without effect on the conditions, which are such as to permit the solution of oxyen in the medium. In the second period characterized by the rapid decline in potential, the bacteria have begun to multiply and in their rapid respiration consume the dissolved oxygen of the medium. With logarithmic growth of the bacteria their respiration becomes sufficient to consume the oxygen more rapidly than it can dissolve into the medium, if the surface of the latter is undisturbed, and the actual concentration of oxygen in the system is reduced to a very low value. The potential at the electrode then shows a negative drift just as in sterile bouillon on removal of oxygen by deaeration, and the potential attained is the same as that found in sterile bouillon under anaerobic conditions.

In the earlier work the equilibrium potential of several specimens of sterile bouillon was found to be -0.060 volt. Different specimens vary somewhat in the value of the reduction potential under anaerobic conditions. Separate determinations have been made of the reduction potentials of the bouillon used in the present investigation, in the sterile condition; the values found are somewhat more negative than those previously reported and have fallen between -0.085 and -0.095 volt. Removal of oxygen by the respiration of the bacteria is thus sufficient to explain the attainment of this level of reduction potential in a growing culture.

That removal of oxygen and not the elaboration of reductive products by the bacteria is responsible for reduction potentials of this intensity in bouillon is indicated by the immediate response of the electrode to small amounts of oxygen. If a few bubbles of air or of pure oxygen are allowed to pass through the culture the potentials at once shift in the direction of more positive values, and if aeration is continued there is a return to the neighborhood of the initial values, or about Eh $+0.200$. On stopping the flow of oxygen there is observed again a negative drift which is more rapid than the initial fall that is observed when the culture contains smaller numbers of living bacteria. When small portions of the culture are withdrawn through the bottom outlet of the cell, the disturbance of the culture is apparently sufficient

to increase the rate at which oxygen is dissolved, since the potentials show a positive shift of 0.050 to 0.100 volt. This phenomenon is observed only when oxygen is available for solution by the culture medium, and does not occur when the medium has been deaerated and the space above the bouillon contains nitrogen.

During the third portion of the time-potential curve the potential is maintained at the value characteristic of the sterile bouillon under anaerobic conditions. The duration of this period is uncertain; it appears to be several hours but probably does not exceed 18 hours from the time of inoculation of the culture. The uncertainty is due to the fact that even in bouillon to which dextrose has not been added there is observed an increase in acidity at the end of 24 hours of 0.2 to 0.3 pH, and the true potentials can be obtained only by a correction of the observed potentials for the change in pH. Frequent sampling for the purpose of following the course of the pH was impossible, because of the effect on the potentials which has been described above. For the same reason it was not possible to follow the growth curve closely during this period. It is known, however, that the growth curve of aerobic cultures of *B. typhosus* becomes flattened after about 18 hours of incubation and the rate of dying of the bacteria begins to approach the rate of multiplication. It is highly probable that autolytic processes occur at this time and liberate products of bacterial metabolism into the culture medium. The gradual fall in potential from about −0.085 volt to values between −0.125 and −0.145 volt which takes place following the brief period of maintained low potential may be regarded as a consequence of this autolysis and as evidence of the reductive nature of the substances set free. The data do not permit further characterization of these products; the potentials for which they appear to be responsible are considerably more negative than those observed in the bouillon when maintained in the sterile condition.

It was anticipated that the addition of dextrose to the bouillon would result in the attainment of more negative potentials by the culture than in a medium without added sugar.[2] This proved not to

[2] The sterile bouillon used in these experiments gave the same equilibrium potential (−0.085 to −0.095 volt) under anaerobiosis when dextrose was added, in the manner described above, to 0.5 per cent concentration as when dextrose was not added.

be the case. Cultures in 0.5 per cent dextrose bouillon given access to a fixed amount of air yielded time-potential curves very similar to those described above, if correction is made for the shift in pH occasioned by

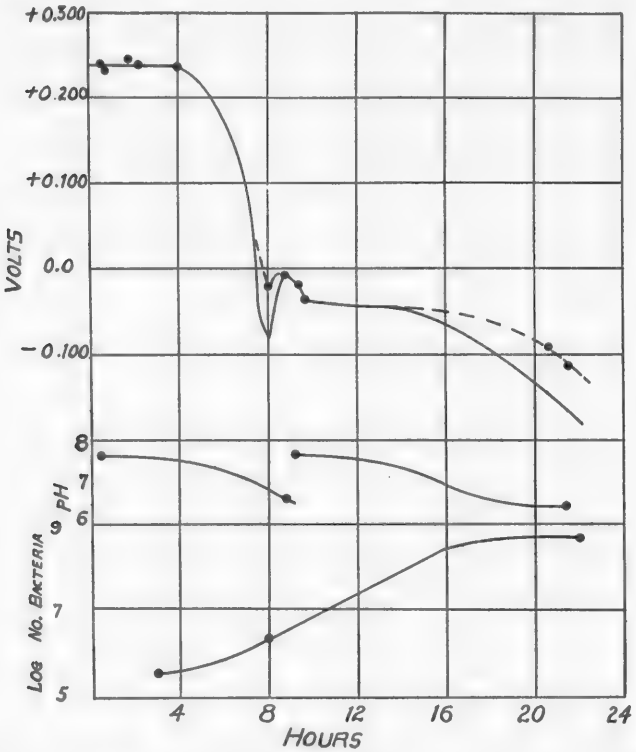

FIG. 2. The upper curve gives the potentials of *B. typhosus* in 0.5 per cent dextrose bouillon. The broken line shows the observed potentials, the solid line the potentials corrected for the change in pH. The positive shift occasioned by sampling of the culture is indicated. The curve of observed potentials represented in part by the broken line was drawn by the recording potentiometer; the experimental points represent values checked by the more sensitive galvanometer. The solid line gives the potentials as corrected for change in pH. The middle curve gives the approximate course of the pH during incubation. The lower curve gives the approximate growth curve. The culture throughout incubation had access to atmospheric oxygen.

acid fermentation. The same effects on the potentials were produced by the admission of air into the culture either deliberately, or incidentally to the disturbance occasioned by sampling, so that the course of reduction potentials and the hydrogen ion concentration could not be followed simultaneously. A typical experiment is shown in Fig. 2. The broken line of the potential curves represents the potentials actually observed; the solid line gives the corrected potentials. The correction of -0.061 volt per unit pH has been applied, on the assumption that no critical points of ionization are uncovered within the range of pH encountered. The potentials show the same rapid drop to more negative values after 6 to 8 hours incubation as in the cultures without added dextrose. The subsequent course of the observed potentials is variable, and in the lack of knowledge regarding the changes in pH, the course of the true or corrected reduction potentials is uncertain. In the experiment recorded in Fig. 2, the pH at the end of 8 hours incubation was 6.6 and the corrected potential was -0.085 volt, which is the value observed at the corresponding period in cultures in which acid fermentation did not occur. At this time the pH of the culture in this experiment was restored to its original value of 7.6 by addition of NaOH. At the end of 22 hours incubation, continued acid production had reduced the pH to 6.7 and the corrected reduction potential was -0.165 volt. This value is 0.040 volt more negative than that observed in cultures without dextrose after the same period of incubation; it suggests that with the increased "turn-over" of bacteria in the presence of dextrose (although the numbers of viable bacteria at any time are no greater than in culture medium without dextrose) a larger amount of reductive substance is liberated from the typhoid bacilli.

The experiments which have been described above indicate that the conditions within an actively growing culture of typhoid bacillus in bouillon are essentially anaerobic, even when the culture has access to atmospheric oxygen. The oxygen which is constantly being absorbed by the culture has an effect on the rate of growth of the bacilli as is shown by experiments which will be reported later, but the actual concentration of oxygen within the medium must be very small. In order to observe the course of the potentials when oxygen is present in high concentration, an experiment was carried out in

which pure oxygen was allowed to bubble through the medium during the period of incubation. The result is shown in Fig. 3, in which the growth curve, and the changes in pH as well as the reduction potentials are recorded. The medium contained 0.5 per cent dextrose. Passage

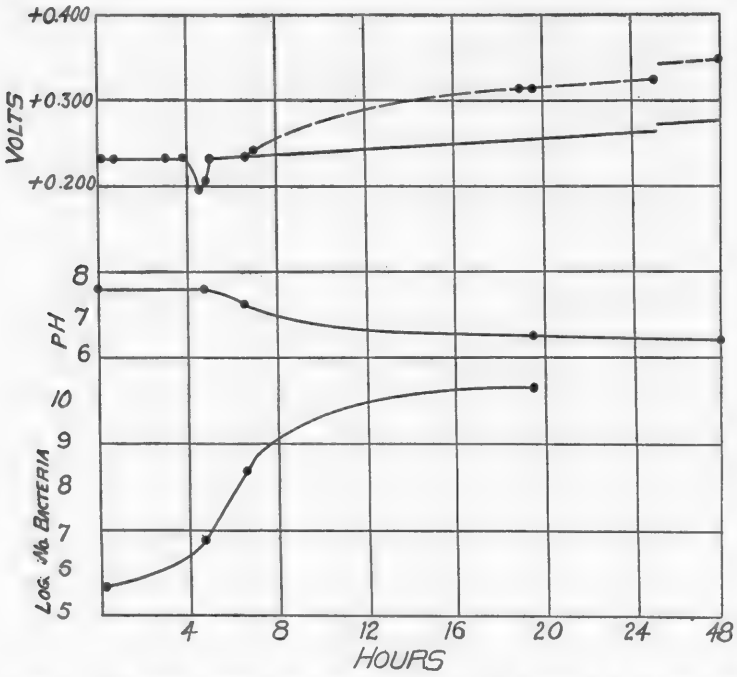

FIG. 3. The upper curve gives the potentials of *B. typhosus* in 0.5 per cent dextrose bouillon. Oxygen was passed through the culture from the time that the early negative drift was observed. The interrupted line represents the observed potentials, the solid line the potentials corrected for change in pH, which is shown in the middle curve. The lower curve represents the growth curve of the bacteria.

of oxygen through the culture was started immediately after the first rapid fall in potential, described above, had begun. The saturation of the medium with oxygen led to an immediate return of the reduction potentials to the initial values. The observed potentials then show a

gradual positive drift; when corrected for the change in pH, a shift to values more positive than those at the beginning of measurement is still evident, although the magnitude of the shift is smaller. The reduction potentials were maintained at a highly positive level throughout 50 hours of incubation. During the first 20 hours of growth the pH shifted from 7.6 to 6.6; the change is less than is observed in cultures which were not aerated. At the same time the numbers of viable bacteria increased enormously. The relations of the oxygen tension to rate of growth and character of metabolism are being made the subject of a separate investigation which will be reported later. In the present connection it is significant that in spite of the enormous numbers of living bacteria, there are not produced reductive substances capable of establishing in the presence of oxygen reduction potentials negative to those of the culture bouillon itself under like conditions. This is not what we should expect if molecular oxygen is inert with respect to reductive substances of bacterial origin. Deaeration at the end of 50 hours incubation, during which saturation of the medium with oxygen had been maintained, led to a rapid drop in reduction potential similar to that observed in cultures which had not been aerated. The equilibrium potential corrected for the shift in pH to 6.4, was − 0.080 volt; this is very close to that observed in the sterile bouillon under anaerobic conditions. Growth of the typhoid bacillus in a high concentration of oxygen appears not to lead to the production of substances of greater reducing intensity than those present in the medium before inoculation; in growth under very low oxygen concentration such substances apparently are produced and are liberated into the medium. Further investigation is required to establish the relation between the character of bacterial metabolism, with respect to the processes of respiration and fermentation, and the elaboration of reductive substances.

DISCUSSION AND SUMMARY.

The reducing properties manifested by bacterial cultures must be regarded as only one phase of a series of processes that come within the scope of the concept of oxidation-reduction phenomena. The importance of such processes is suggested in many phases of the metabolism of living cells, as in the accumulation of carbohydrate within

the cell, and the elaboration of other readily oxidized substances such as the hemotoxins studied recently by Neill (9). In these cases the energy of the living cell transforms substances in a state of relatively high oxidation into bodies that are in a condition of low oxidation. Conversely in respiration, as in the familiar oxygen-carbon dioxide exchange, a change in the condition of oxidation of carbon takes place in the opposite direction.

The scope of this concept is therefore very broad and includes other processes than the reduction of dyes and other substances such as nitrates and sulfhydryl bodies. Some definite aspect of the general problem must be chosen in order to bring the subject within the range of experiment. In the present series of investigations attention is centered upon those processes of oxidation and reduction which involve electromotively active substances, and are therefore subject to electrical measurement.

In the present paper the oxidation-reduction potentials yielded by cultures of the typhoid bacillus have been observed under the conditions which prevail in the usual aerobic culture in bouillon. Comparison of these potentials with those given by the culture medium in the sterile condition indicates that the living bacteria do not contribute the substances which are responsible for the observed potentials. On the other hand the respiratory consumption of oxygen by the typhoid bacillus appears to render possible the manifestation of characteristic potentials by the culture medium. Such potentials become apparent only when oxygen is removed from the system, and the rôle of the bacteria is the establishment of the anaerobic state.

The potentials of cultures in which the anaerobic condition was maintained from the beginning by artificial means will be reported in a later paper, but it may be said here that the potentials of such cultures confirm the idea of the importance of bacterial respiration in establishing a condition of anaerobiosis in cultures given access to air. The anaerobic cultures develop potentials of the same value as those in which oxygen is available, but attain the level characteristic of the culture medium more rapidly.

This point of view is opposed to that which regards the potentials of bacterial cultures as a manifestation of reductive substances liberated by the bacteria. Since the potentials given by cultures in the

early part of their growth indicate only the level of reduction intensity which the sterile medium may attain, evidence is wanting from this source for the existence of such bacterial products.

In the later stages of growth when the rate of dying of the bacteria approaches the rate of multiplication, the potentials of cultures indicate a level of reduction intensity more negative than that of the culture medium itself. No attempt has been made to work out the exact relations between the growth curve of the bacillus and the reduction potentials since this would involve the detailed investigation proposed by Clark (3). It is certain however that reductive bodies are produced and stored within the cell, as is shown by the reduction of indicators within the living ameba in the experiments of Cohen, Chambers, and Reznikoff (10). The dissolution of cells which must occur in bacterial cultures that have passed the peak of the growth curve would be expected to liberate such reductive substances; if their oxidation is prevented, as in the anaerobic condition which results from respiration of the bacteria, and if in addition the substances are electromotively active, their presence might be indicated by change in the reduction potentials.

It seems possible from consideration of the potentials of cysteine studied by Michaelis and Flexner (6) that among the substances liberated on dissolution of the bacteria are products similar in nature to those already present in the culture medium, so that the more negative potentials may result from an *increase* in the concentration of an electromotively active substance. Other reductive products may be liberated, but nothing can be said at the present time as to their effect on the electrode potentials.

If the substances responsible for reduction potentials more negative than those of the culture medium itself were secreted during growth of the bacteria, rather than liberated by their dissolution, we should expect that their effect on the potentials would be observed during the youth of the culture. The gradual negative drift to values 0.040 to 0.080 volt more negative than those of the sterile bouillon which is observed in cultures after several days incubation indicates that distinct reductive phenomena are associated with the declining portion of the growth curve, in which death of the cells predominates over multiplication.

The failure to attain reduction potentials in cultures saturated with oxygen as negative as those given by cultures which were allowed to develop an anaerobic state may be due to the oxidation of any highly reductive bodies which had been liberated. On the other hand, the enormous numbers of living bacteria in the oxygenated culture suggest that there may be a less rapid "turn-over," and consequently a less extensive dissolution of bacterial cells in such cultures than in those provided with a less abundant supply of oxygen.

The behavior of the typhoid bacillus in the establishment of reduction potentials does not of course permit inference to be drawn as to the behavior of other bacterial species. The observations of Clark (3) show that different species run different courses of potential. It is probable however that any microorganism capable of consuming oxygen as completely as the typhoid bacillus would bring about in bouillon the establishment of potentials as negative as those yielded by the medium itself under anaerobic conditions, unless there is involved an oxidative mechanism which is not evident in the case of *B. typhosus*.

CONCLUSIONS.

1. The reduction potentials of *B. typhosus* in culture in bouillon which is given access to atmospheric oxygen show a negative drift that attains the values found in sterile bouillon when deaerated with nitrogen: *Eh* −0.085 to −0.095 volt at pH 7.6. The potential reaches this level after 6 to 8 hours incubation, and is maintained at this point for several hours. A slow decline to more negative values is then observed and continues for at least 48 hours, when a potential of −0.145 volt may be attained.

2. The bacteria influence the potentials in the first period of their growth by exhaustion of oxygen from the culture, thus permitting the characteristic potential of the culture medium to become manifest, and do not contribute the substances responsible for the observed potentials. The decline in potential to values more negative than those of the culture medium occurs during the time that the rate of dying of the bacteria approaches and exceeds the rate of multiplication; it is suggested that dissolution of bacteria liberates reductive substances.

3. Cultures in 0.5 per cent dextrose medium show a somewhat more negative potential after 18 hours growth than cultures in medium without dextrose. This may be due to the more rapid "turn-over" of the bacteria and the liberation of larger amounts of reductive material from dissolution of larger numbers of bacteria.

4. The potential of cultures through which oxygen is passed continuously does not show a negative drift at any time. This indicates that reductive substances of bacterial origin in the case at least of the typhoid bacillus do not influence the electrode potentials in the presence of oxygen and confirms the importance of bacterial respiration as the means for the removal of oxygen and the consequent establishment of characteristic reduction potentials in cultures.

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STUDIES ON THE PRECIPITABLE SUBSTANCES OF BACILLI OF THE SALMONELLA GROUP.

By J. FURTH, M.D., AND K. LANDSTEINER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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In earlier communications we (1) have described precipitable substances, probably of carbohydrate nature, isolated from *B. typhosus*, *paratyphosus* B, and *enteritidis*, and two antigenic protein preparations obtained from *B. typhosus*. These studies have also been extended to the main serological types of the Salmonella group of organisms.

For a general review of the subject we refer to the communications by P. Bruce White (2), Krumwiede, Cooper, and Provost (3), Tulloch (4), and to the references given in our earlier papers.

We would however like to mention several recent publications. Thus Happold (5) described a precipitable substance obtained from *B. aertrycke* which he considered to be of protein nature and "to be identical with the antigen which stimulates the production of agglutinins to a heat-stable antigenic form of the organism." Ecker and Rimington (6) report obtaining from *B. aertrycke* a carbohydrate containing material possessing toxic properties; while White (7) states that he has extracted a soluble specific substance from *B. aertrycke* similar to the specific soluble substances of Avery and Heidelberger. The precipitable solution obtained was acted upon by the sera of *B. aertrycke* and *paratyphosus* B but not by those of *B. Newport* and *suipestifer*. White's paper contains a full discussion of the serological properties of the rough *B. aertrycke* strains. Casper (8) prepared from *B. paratyphosus* B a carbohydrate containing substance which reacted with sera for *B. paratyphosus* B, *B. typhosus*, and *B. typhi murium*, to a lesser degree with sera for *B. enteritidis* Gärtner, but not with *suipestifer* serum. A report on the carbohydrate and protein fractions of *B. typhosus* was made by Heidelberger, Shwartzman, and Cohn (9).

Preparation of the Crude Precipitable Carbohydrate Substances.—Three methods were employed for the preparation of the crude substances:

1. Extraction by alkaline hypochlorite solution: Bacilli grown on agar for 48 hours were taken up in 0.9 per cent sodium chloride solution, and alkaline hypochlorite solution was added in a quantity sufficient to dissolve the bacteria at about 50°C. The solution was chilled and cold 95 per cent alcohol added until a heavy precipitate was formed carrying down most of the active substance. This

TABLE I.

Precipitation Tests.

Carbohydrates from *Bacillus*	Antigen dilutions	Immune sera obtained with *Bacillus*											
		Typhosus		*Enteritidis*		*Paratyphosus* B		*Derby*		*Newport*		*Hog cholera*	
Typhosus	5,000	+++	+++	++	++++	+	±*	++	+++	0	0	0	0
	50,000	+±	++±	+	++	±	+	+	+	0	0	0	0
	500,000	±	±	f. tr.	±	0	0	0	tr.	0	0	0	0
Enteritidis	5,000	+±	+++	++	++++	tr.	±	+	+	0	0	0	0
	50,000	±	±	+	++	0	0	0	tr.	0	0	0	0
	500,000	0	tr.	f. tr.	±	0	0	0	0	0	0	0	0
Paratyphosus B	5,000	tr.	±	+±	++	+±	++	+++	++++	0	0	0	0
	50,000	0	0	±	+	+	+++	++	++±	0	0	0	0
	500,000	0	0	0	±	±	+	±	±	0	0	0	0
Derby	5,000	±	+	+	++	+±	+++	++++	++++	0	0	0	0
	50,000	f. tr.	±	±	+	+	+++	++	++±	0	0	0	0
	500,000	0	0	0	tr.	±	+	±	±	0	0	0	0
Newport	5,000	0	0	0	0	0	0†	0	0	++	++++	tr.	±†
	50,000	0	0	0	0	0	0	0	0	+	+++	0	0
	500,000	0	0	0	0	0	0	0	0	±	+	0	0
Hog cholera	5,000	0	0	0	0	0	0	0	0	+	+++	+++	++++
	50,000	0	0	0	0	0	0	0	0	±	++	++	++++
	500,000	0	0	0	0	0	0	0	0	tr.	++	+	+±

To 0.1 cc. of the antigen solution 0.05 cc. immune serum and 0.05 cc. physiological salt solution were added. The reactions were read after 2 hours at room temperature (Column 1) and after standing overnight in the ice box (Column 2).

The immune sera were obtained by injections of rabbits with heated bacilli, as stated above.

* With other sera of the same type the reactions were negative with the substance of *B. typhosus*.

† With some other sera of the same type marked reactions were obtained with the substance of *B. Newport*.

precipitate was dissolved in water, and, after removal of some insoluble material, was reprecipitated with 95 per cent alcohol. After washing with 95 per cent alcohol, absolute alcohol, and ether, it was dried *in vacuo*.

2. Extraction with saline solution: Bacilli grown on agar for 48 hours were taken up in 0.9 per cent sodium chloride solution, centrifuged,[1] the sediment washed with 95 per cent alcohol, and extracted with boiling 95 per cent, and absolute, alcohol. After filtration on a hot water funnel the bacillary mass was heated in the steam bath two or three times with 0.9 per cent sodium chloride solution for 1 to 2 hours and the extract separated each time by centrifuging. Much of the protein was removed by addition of hydrochloric acid in an amount sufficient to produce maximum precipitation and the fraction was reprecipitated to recover some of the active non-protein substance; the joint mother liquids were then precipitated with alcohol after addition of 1/20 volume of normal sodium hydroxide. The further redissolving, removal of insoluble material, reprecipitation, and drying of the preparation were done as described above.

3. Extraction by autoclaving and subsequent tryptic digestion (*cf.* Goebel and Avery (10)): Agar cultures were washed off and were autoclaved for 20 minutes and digested overnight with purified trypsin solution (Goebel and Avery) at 37°C. The subsequent alcohol precipitation, removal of insoluble material, reprecipitation, and drying of the substance were carried out in the manner given.

Cultures.—Besides our laboratory strains the following cultures obtained through the courtesy of the National Collection of Type Cultures, London,[2] were used: *B. paratyphosus* B, *Tidy No. 14;* type *Stanley, No. 92;* type *Reading, No. 72;* type *Derby, No. 1729;* type *Newport, No. 129;* type *Hog cholera, No. 356; B. enteritidis, Gärtner, No. 127; B. abortivo-equinus, No. 766;* and *B. aertrycke.*[3] They were plated on agar and colonies showing homogeneous growth in broth were selected.

Immune Sera.—Unless otherwise stated, immune sera were prepared by weekly injections of rabbits with about 1/10 to 1/20 of 24 hour agar slant cultures heated to 62–65°C. for 40 minutes. Immune sera prepared with various strains of the same type, *e.g. B. typhosus*, showed considerable variations in their capacity to precipitate the carbohydrate preparations.

Precipitin Reactions of the Carbohydrates and Their Parallelism to the "Small Flaking" Agglutination.—Precipitin reactions with the purified specific substances (see page 737) are presented in Table I. The crude substances had a lower titer but showed in cross-tests almost the

[1] In a control experiment the bacillary mass was washed several times with saline solution.

[2] We are indebted to Dr. R. St. John-Brooks, Curator of the National Collection of Type Cultures, London, for supplying us with the cultures mentioned.

[3] The numbers correspond to those given in the catalogue of the collection.

same range of specificity as purified preparations, no matter which of the methods described was employed.

It is seen that the strains can be divided roughly into three groups within which the strongest cross-reactions occur. The first group consists of *B. typhosus* and *B. enteritidis*, the second of *B. paratyphosus* B, and *B. Derby*, and the third of *B. Newport* and *B. hog cholera*. Additional tests with crude preparations, not included in Table I, showed that the substances and sera of *B. Stanley*, *B. Reading*, and *B. abortivo-equinus* reacted like those of *B. paratyphosus* B.

TABLE II.*

Typhosus	*Enteritidis*	*Paratyphosus* B	*Derby, Reading, abortivo-equinus*	*Newport*	*Hog cholera*
III, (X), 8	III, 8	I, II, 7, 8	II, 7, 8	IV, VI, 7	V, VI

* Roman numerals are used by White to designate salient components, Arabic numerals for those of minor development.

TABLE III.

Absorption Tests with Immune Serum for B. typhosus.

Dilution of the carbohydrates	Serum, unabsorbed		Serum absorbed with alcohol-treated *B. enteritidis*	
	Precipitation tests with the carbohydrate of *B. typhosus*			
5,000	++	+++	0	0
50,000	++	++	0	0
500,000	f. tr.	tr.	0	0
Dilution of the immune serum	**Agglutination tests with *B. typhosus* (suspension preserved with chloroform)**			
300	+++	+++	+++	+++
900	+++	+++	++	++
2,700	++	++	+±	+±
8,100	+	+	±	tr.
24,300	0	tr.	0	0

The reactions were read after 1½ hours at room temperature (Column 1) and after standing overnight in the ice box (Column 2).

A comparison of these results with the agglutinin reactions of the "stable agglutinogens" suggested itself. The distribution of the

agglutinable factors underlying the "small flaking" agglutination is summarized, according to White, in the scheme shown in Table II.

On comparing this scheme with the precipitin reactions an approximate correspondence between the stable agglutinogens and the precipitable carbohydrates appears (see White).

This relation is also illustrated by the following observations (Table III): An immune serum for *B. typhosus* was absorbed with alcohol-treated *B. enteritidis* and tested for precipitins and agglutinins to *B. typhosus*. Since the "stable" alcohol-resistant agglutinogens of *B.*

TABLE IV.

Absorption Experiments.

Dilution of the precipitable substance	Precipitable substance	Immune serum for *B. enteritidis*		Precipitable substance	Immune serum for *B. Newport*		Precipitable substance	Immune serum for *B. hog cholera*	
		Unabsorbed	Absorbed with *B. paratyphosus* B		Unabsorbed	Absorbed with *B. hog cholera*		Unabsorbed	Absorbed with *B. Newport*
2,000	*Enteritidis*	+++	+++	*Newport*	+++	+++	*Hog*	+++	+++
20,000		+++	+++		++±	++±	*cholera*	+++	+++
200,000		±	±		+	+		++±	++±
2,000	*Paraty-*	+++	0	*Hog*	++	0	*Newport*	+	0
20,000	*phosus* B	+++	0	*cholera*	+++	0		±	0
200,000		+±	0		++±	0		tr.	0

The immune sera were absorbed with bacillary suspensions kept in alcohol and washed once with saline solution.

typhosus and *B. enteritidis* are supposed to be very similar it was to be expected that by the exhaustion with alcohol-treated *B. enteritidis* the precipitins would be removed but not the "large flaking" agglutinins. The result of the experiment bore out this assumption.

In contrast with the absence of precipitating action of fluids with large flaking agglutinins of high titer (*cf.* Heidelberger, Shwartzman, and Cohn) are instances in which immune sera with a relatively low agglutinin content, mainly of the small flaking type, precipitated intensely the carbohydrate solutions. Such sera were prepared by the method of Douglas and Fleming (1).

Some discrepancies will be noted such as the absence of precipitin reactions of certain *B. paratyphosus* B sera on the substances of *B. Newport* (factor 7) and *B. typhosus* (factor 8), and the lack of precipitating capacity of most of the hog cholera sera for the substance derived from the strain *Newport* (factor VI). Since in these combinations some of the sera give positive reactions one may assume that the precipitable substances do not lack the properties in question. An explanation may perhaps be seen in the observation that the agglutinin reactions with alcohol-treated bacilli were comparatively weak in

TABLE V.

Precipitable substance	Dilution	Immune serum for *paratyphosus* B				
		Unabsorbed, diluted			Absorbed with *B. Reading*, diluted	
		1:2	1:8	1:32	1:2	1:8
Paratyphosus B, not treated with alkali	5,000	+++	+++	+	++++	+++
	50,000	+±	++	++	++	++
	500,000	±	±	tr.	±	−
Paratyphosus B, treated with alkali	5,000	++	+	tr.	0	−
	50,000	+	+	0	0	−
	500,000	f. tr.	0	0	0	−
Reading (treated with alkali)	1,000	+++	++	tr.	0	−
	5,000	++	+±	f. tr.	0	−
	25,000	+	+	f. tr.	0	−

those instances in which precipitation was lacking. However, a thorough study of this question was not made.

In order to determine whether the multiplicity of precipitins corresponds to that of the agglutinins, absorption experiments were made in several cases (Table IV).

On the whole the tests were in agreement with the idea of a multiplicity of precipitins, corresponding to that of agglutinins. A striking exception was the following:

Immune sera for *B. paratyphosus* B or *B. Stanley*, when absorbed with *B. Reading* or *B. abortivo-equinus* did not precipitate the substances prepared from the homologous organisms, although they agglutinated the alcohol-treated bacillary suspensions. Search for

the missing precipitable property (attributable to factor I) revealed that it was present in the crude saline extracts, but disappeared after treatment with alkali. Substances exhibiting the property were prepared as follows:

Saline extracts obtained from alcohol-extracted bacilli, as described above, were precipitated with alcohol (without the addition of alkali), after previously removing the substances precipitable by dilute acid. The precipitate was dissolved in a small volume of water, some insoluble material discarded, and 50 per cent trichloracetic acid added to cause optimal precipitation. The unneutralized mother liquid containing most of the active substance was then precipitated with an excess of 95 per cent alcohol and dried after washing several times with 95 per cent alcohol, absolute alcohol, and ether.

Table V indicates that immune sera for *B. paratyphosus* B (or *B. Stanley*) contain a fraction of antibodies directed towards the alkali-labile property I. After absorption with *B. Reading* the immune serum for *B. paratyphosus* B still precipitates the substances derived from this bacillus but no longer acts on the substance from *B. paratyphosus* B after treatment with alkali. That this effect is not due to a diminution of one single antibody was shown by tests with diluted immune serum.

The reactivity of the substance was not altered by peptic and tryptic digestion at a pH not in itself injurious. It was not destroyed by treatment for 1 hour at room temperature, with normal hydrochloric acid, but it did not withstand under the same conditions the action of 0.01 normal sodium hydroxide (Table VI).

A preliminary investigation was made of the carbohydrates of "rough" strains of *B. paratyphosus* B and *B. aertrycke*. According to several authors such strains may contain special antigenic components. In view of these statements we prepared precipitable substances from "rough" strains by the method of dissolving the bacilli in alkaline hypochlorite solution (see page 727). The substance prepared from *B. aertrycke* "R" and purified as described below proved to contain large amounts of carbohydrates not further investigated. In precipitation tests (Table VII) the "R" substances reacted specifically with the immune serum to the "R" strains whereas the corresponding "S" preparations reacted also to "R" immune sera. It is uncertain whether the interaction can be explained by the presence of "R" forms in the "S" strains.

Attempts to Fractionate the Precipitable Substances by Means of Precipitins.—The resistance of the specific precipitable substances to the action of alkali seemed to offer a way of ascertaining whether the various serological properties of these substances are due to different

TABLE VI.

Precipitin Tests Showing the Effect of Acid, Alkali, Peptic, and Tryptic Digestion on Factor I of B. paratyphosus B.

Dilution of the precipitable substance	Control I	0.01 N NaOH	N HCl	Peptic digestion	Control II	Tryptic digestion I	Tryptic digestion II	Control III
5,000	++++	0	+++	+++	+++	+++	0	0
100,000	+	0	±	+	+±	+	0	0

To 0.1 cc. of the precipitable substance, 0.1 cc. of an antibody solution for factor I (immune serum to *B. paratyphosus* B absorbed with suspensions of *B. Reading*) was added.

Control I: untreated precipitable substance.

Control II: 0.5 cc. of a 1 per cent solution of the substance, 0.05 cc. N HCl, 0.45 cc. water; kept at 37° for 24 hours.

Control III: 0.5 cc. of a 1 per cent solution of the substance, 0.2 cc. of a 1 per cent solution of Na_2CO_3, 0.8 cc. water; kept at 37° for 24 hours.

Peptic digestion: 0.5 cc. of a 1 per cent solution of the substance, 0.05 cc. N HCl, 0.1 cc. of a 0.2 per cent pepsin solution, 0.35 cc. water; kept at 37° for 24 hours.

Tryptic digestion I: 0.5 cc. of a 1 per cent solution of the substance, 0.5 cc. of a 1 per cent trypsin solution, 0.05 cc. 1 per cent Na_2CO_3, 0.45 cc. water; kept at 37° for 24 hours.

Tryptic digestion II: 0.5 cc. of a 1 per cent solution of the substance, 0.5 cc. of a 1 per cent trypsin solution, 0.2 cc. 1 per cent Na_2CO_3, 0.3 cc. water; kept at 37° for 24 hours.

For the treatment with acid and alkali a 2 per cent solution of the substance was mixed with an equal volume of 0.02 N NaOH or 2 N HCl respectively and the tests kept for 1 hour at room temperature.

separable portions. Accordingly attempts were made to fractionate the precipitable substances in a manner analogous to the usual absorption experiments with immune sera.

By suitable exhaustion with heterologous bacilli fractions of immune sera were prepared acting only on part of the supposed precipitable factors. The precipitates produced were washed twice with saline

solution and boiled for a few seconds with a small quantity of 0.1 normal sodium hydroxide solution, the solutions obtained being neutralized and centrifuged.

When such solutions were tested with various antibodies they behaved like the original precipitable substance and not like qualitatively different fractions thereof.[4] With the procedure described no difficulty was encountered in separating precipitable substances of two different strains of bacilli after their solution had been mixed. It was

TABLE VII.

Precipitation Test.

Immune serum of *Bacillus*.....		*Aertrycke* "S"		*Aertrycke* "R"		*Paratyphosus* B "S"		*Paratyphosus* B "R"	
Precipitable substance	Dilutions								
Aertrycke "S"	2,000	+++	+++	+±	++±	+±	+±	+±	+++
	20,000	++	++	±	++	+	++	tr.	+
	100,000	±	+	f. tr.	+	±	+±	0	tr.
	500,000	0	0	0	0	0	0	0	0
Aertrycke "R"	2,000	0	0	+±	++±	0	0	++	++±
	20,000	0	0	0	0	0	0	tr.	+
Paratyphosus B "S"	2,000	+++	+++	++	++±	+	++	++	++
	20,000	++	++±	+	++	±	++	tr.	+±
Paratyphosus B "R"	2,000	0	0	++	+++	0	0	+++	+++
	20,000	0	0	++	+++	0	0	++	++±

Reactions read after 2 hours (Column 1) and after standing overnight (Column 2).

thought however that in these control experiments the mixture of the substances might not have been as intimate as that of the hypothetical fractions in the precipitable carbohydrates derived from one organism. We attempted therefore to separate by the same method precipitable substances after their solutions had been mixed, boiled, precipitated with alcohol, and dried in the usual manner.

[4] The results were the same in experiments aiming at the separation of Factors I and II of the precipitable substances of *B. paratyphosus* B. In this instance, instead of alkali, dilute acid was used for decomposing the precipitate.

TABLE VIII.

Precipitable substance	Dilution	Immune sera for:							
		B. paratyphosus B absorbed with *B. enteritidis* (II-7)		*B. enteritidis* (8)		*B. hog cholera* absorbed with *B. Newport* (V)		*B. Newport* (VI)	
Mixture of the carbohydrates of *B. paratyphosus* B and *B. hog cholera*	1,000	+±	++++	+	++++	++	++++	++	++++
	10,000	++	++++	±	+±	+±	+++	+±	++++
	100,000	tr.	+	f. tr.	±	f. tr.	tr.	f. tr.	tr.
Factor II, 7	1,000	+±	+++	+	+±	0	0	0	0
	10,000	±	+±	tr.	±	0	0	0	0
	100,000	0	0	0	0	0	0	0	0
Factor 8	1,000	+±	+++	+	++	0	0	0	0
	10,000	tr.	tr.	0	f. tr.	0	0	0	0
	100,000	0	0	0	0	0	0	0	0
Factor V	1,000	0	tr.	0	0	+++	++++	++±	++++
	10,000	0	0	0	0	±	+	±	+±
	100,000	0	0	0	0	0	tr.	0	tr.
Factor VI	1,000	0	f. tr.	0	0	++	++±	+±	++±
	10,000	0	0	0	0	±	+	±	++
	100,000	0	0	0	0	0	0	0	0

To a 0.1 per cent solution of the mixture of *B. paratyphosus* B and *B. hog cholera* carbohydrates an equal volume of the immune sera (diluted 1:2) was added. After keeping the tubes for 2 hours at room temperature and overnight in the ice box, the precipitate formed was dissolved by sodium hydroxide, as described, and brought with saline solution to half of the original volume. (This dilution was arbitrarily designated 1:1000.)

Factor II, 7: precipitate formed by *paratyphosus* B immune serum absorbed with *B. enteritidis*.

Factor 8: precipitate formed by immune serum of *B. enteritidis*.

Factor V: precipitate formed by hog cholera serum absorbed with *B. Newport*.

Factor VI: precipitate formed by immune serum of *B. Newport*.

In these tests a considerable amount of carbohydrate was carried down non-specifically by the heterologous serum. The possibility that this effect was caused by imperfect solution of the mixture of precipitable substances led to the following modification of the experiment. The dried mixture obtained as before was dissolved in 0.1 normal sodium hydroxide, boiled for 1 hour, and neutralized. In this way again by specific precipitation an almost complete separation could be brought about.

In the following experiment this method was applied to the separation of fractions of single precipitable substances (Table VIII).

The experiment shows that while the two substances mixed together could be separated, apparently no fractionation of either of them was accomplished.

An analogous experiment was carried out with the substance of *B. Newport* and with the sera to *B. suipestifer* (factor VI), *B. paratyphosus* B (factor 7), and *B. Newport*, after exhaustion with *B. suipestifer* and *B. paratyphosus* B (factor IV). Comparing factors IV and VI the results resembled those just reported; the substance however precipitated by *paratyphosus* B serum gave a considerably stronger reaction with this serum than with the hog cholera serum. Conversely the solution obtained from the precipitate caused by the hog cholera serum reacted, like the original substance, more intensely with this serum than with *B. paratyphosus* B serum.

Chemical Data on the Precipitable Substances.—Several of the substances described were purified to a certain extent by a procedure very similar to that used for pneumococcus polysaccharides by Avery, Heidelberger, and Goebel (11).

The crude preparations were dissolved in water, some insoluble matter removed, and, after addition of normal sodium hydroxide to a concentration of about N/20, the active substance was precipitated with alcohol. This precipitation was repeated 4 to 6 times. Usually sodium acetate was added to aid flocculation. Then the solution was acidified with hydrochloric acid and precipitated with acidulated alcohol. When possible the precipitate was made in two steps, the first precipitate, containing most of the proteins and much carbohydrate, was removed by centrifugalizing and the supernatant fluid poured into an excess of acidulated alcohol. From the first pre-

cipitate, rich in proteins, part of the carbohydrate could be recovered by repeating the procedure.

The carbohydrate substance was reprecipitated in acid solution and dried after washing with alcohol and ether. The solutions were kept at low temperature while in acid solution.

These fractions were soluble in water, strongly reduced Fehling's solution after hydrolysis, and gave faint or negative protein reactions, particularly no precipitate with tannic acid or uranyl nitrate. The purified substance of *B. typhosus*, unlike the crude preparation, was not precipitated by barium hydroxide.

On analysis, the figures for the N content of the substances prepared from *B. typhosus*, *B. enteritidis*, *B. paratyphosus* B, *B. Derby*, *B. Newport*, and *B. hog cholera*, varied from 0.5 per cent to 1.4 per cent. The values for $[\alpha]^D$ were as follows: *B. typhosus*, +103; *B. enteritidis*, +95; *B. paratyphosus* B, +94; *B. Derby*, +76; *B. Newport*, +75; *B. hog cholera*, +48. For the sugar analysis the substances were hydrolyzed by heating 1 per cent solutions with an equal volume of normal hydrochloric acid in the steam bath for 5 hours, and the sugar content was determined by reduction of Fehling's solution. It was not established whether the time chosen was sufficient for complete hydrolysis. The values obtained were between 63 per cent and 74 per cent (calculated as glucose) for the six strains mentioned. During hydrolysis with acid some insoluble material, partly soluble in alcohol and of acid character, separated from the solutions as in the case of the specific substance from *V. choleræ* (12). No conclusion can be reached as yet whether these products form a part of the precipitable substances or are impurities.

Since it seemed possible that the precipitable substances contained carbohydrates derived from the agar used for cultivating the bacteria, two preparations were made from gelatin cultures of *B. typhosus* and *B. paratyphosus* grown in Blake bottles. They gave the following figures: *B. typhosus*, reducing sugar 69.5 per cent, $[\alpha]^D + 98°$; *B. paratyphosus*, reducing sugar 67.3 per cent, $[\alpha]^D + 99°$. It cannot be claimed that the substances were obtained in a state of purity and therefore all of the figures given are to be considered as preliminary.

On testing the action of alkali and acid it was found that the precipitable substances were remarkably resistant to alkali but readily destroyed by acids.

TABLE IX.

Precipitin Tests with the Carbohydrates after Treatment with Acid and Alkali.

Carbohydrates		Heating with N HCl for				Heating with N NaOH for				Control untreated	
From *Bacillus*	Dilution	3 min.		15 min.		½ hr.		2 hrs.			
Proteus O_{X19}	10,000	0	f. tr.	0	f. tr.	0	0	0	0	+++	+++
	100,000	0	0	0	0	0	0	0	0	+	+±
V. choleræ	10,000	±	+++	0	0	0	+++	0	+	++	+++
	100,000	0	++	0	0	0	±	0	0	±	+
Pneumococcus Type III	10,000	++	++	+±	+±	++	++	++	++	++±	++±
	100,000	+	+±	±	+	±	+	±	+	+	+±
B. typhosus	10,000	0	0	0	0	–		++	+++	+++	+++
	100,000	0	0	0	0	–		±	++	+	++
B. enteritidis	10,000	0	0	0	0	–		++±	++±	++±	+++
	100,000	0	0	0	0	–		±	+±	+	+±
B. paratyphosus B	10,000	0	f. tr.	0	0	–		+±	++±	++	++±
	100,000	0	0	0	0	–		++	++±	++	++
B. Derby	10,000	0	0	0	0	–		+++	+++	+++	+++
	100,000	0	0	0	0	–		+	+±	+	+
B. Newport	10,000	0	0	0	0	–		++	+++	++	+++
	100,000	0	0	0	0	–		+	++	+	++
B. hog cholera	10,000	+±	++±	0	f. tr.	–		++±	+++	+++	+++
	100,000	0	+±	0	0	–		+±	++	+±	++

The substances of *Proteus* O_{X19} and cholera were prepared by extraction with boiling 75 per cent alcohol (1,10). The specific polysaccharide of Pneumococcus III was obtained through the courtesy of Dr. Avery.

The first reading of the precipitin tests was made after 2 hours at room temperature, the second reading after the tests were kept overnight in the ice box.

To a 2 per cent solution of the various preparations an equal volume of 2 normal sodium hydroxide or 2 normal hydrochloric acid was added and the solutions were kept in boiling water.

It is seen from the experiment (Table IX) that the preparations of the *typhosus-paratyphosus* group were similar as regards their resistance to acid and alkali, with the exception of the hog cholera substance which was somewhat more resistant to acid than the other preparations. The substances from the other organisms tested, behaved differently. Thus the preparation obtained from *Proteus* O_{X19} was destroyed by alkali as well as by acid, under the conditions of the experiment; the Pneumococcus III preparation was resistant to both, whereas the substance derived from *V. choleræ* was less resistant to alkali than the Salmonella carbohydrates. The differences found would seem to be significant even if one takes in account the variation in the method of preparation.

Observations on the Precipitable Proteins of B. typhosus.—A precipitable protein was prepared in a similar manner as the preparation P_2 *typhosus* described previously (1). The precipitation with alcohol of the extracted substance was omitted but after removal of the suspended bacilli by centrifugalizing, the saline extract was precipitated with dilute hydrochloric acid, redissolved by addition of a small quantity of alkali, filtered through a Berkefeld filter, and reprecipitated with acid. The precipitate was dried after washing with alcohol and ether.

This substance when injected into rabbits induced the formation of "large" and "small" flaking agglutinins aside from precipitins. Although this would seem to point to a relation between the substance P and "large flaking" agglutinogens there are observations which do not agree with this assumption. In the first place the titer of the large flaking agglutinins was relatively low in comparison with sera obtained with bacillary suspensions, and on prolonged immunization the increase of precipitins was not accompanied by a corresponding rise in the agglutinin titer. Furthermore in an absorption experiment the large flaking agglutinins of the sera for P_2 were apparently absorbed to a greater extent by typhoid bacilli treated with alcohol than the large flaking agglutinins of common typhoid sera.

It is as yet difficult to interpret these observations. They may possibly be ascribed to the presence in the P_2 preparations of a special substance responsible for the production of flagellar agglutinins, or to some flagellar material perhaps in an altered state which passed the filter candles. In this respect attention may be called to the observation that bacilli treated with alcohol are no longer agglutinable by large

flaking sera although they give rise to the formation of large flaking agglutinins (13).

The toxic action of the preparation P_2, mentioned previously, showed considerable variation. One preparation was toxic for rabbits in a dose of 0.5 mg. given intravenously. On repeated injections the animals tolerated doses up to about 20 mg. These animals as well as those immunized with digested bacilli (14) exhibited a typical Arthus phenomenon on intradermal injections of about 1 mg. of P_2 or the carbohydrate respectively. In cross-tests the reactions with the homologous substances were more pronounced. (*Cf.* the experiments on anaphylaxis by Tomcsik (15) and Avery and Tillett (16).) These tests were made on a small number of animals and therefore should be considered as preliminary.

DISCUSSION.

The carbohydrate-containing preparations isolated from the main serological types of the Salmonella group gave on analysis figures for nitrogen of 0.5 to 1.4 per cent, but they showed only weak or negative reactions for proteins. One may assume that their serological activity is due to specific carbohydrates, for during purification there was a diminution of the nitrogen content and of the protein reactions along with an increase in the amount of sugar liberated by hydrolysis and an increase of the serological activity. The specific reactivity of the preparations to immune sera remained almost unimpaired after heating to about 100° with normal alkali for 2 hours but was quickly destroyed by boiling with normal hydrochloric acid; parallel with the disappearance of the serological activity reducing sugar and some insoluble material were set free. Aside from these observations the assumption that the substances are carbohydrates rests on the analogy of the results with those of Avery, Heidelberger, and Goebel (*cf.* 11).

The present studies support the view that the specific carbohydrates form an essential part of the "stable" agglutinogens of the bacilli (White (7)). We failed to establish a relationship to the phenomenon of large flaking agglutination.

The similarity of agglutination and precipitation is also shown by absorption experiments, which demonstrate that from one immune serum precipitins can be separated which correspond to agglutinin fractions (*cf.* Krumwiede (17)).

The explanation of these phenomena so far as the antigens are concerned is still an open one. The fact that certain antibodies are removed from an immune serum through successive absorptions and others are left behind gives evidence for a multiplicity of antibodies; but when the conclusion is drawn from the reaction of certain antibody fractions upon several antigens, that all of the positively reacting antigens contain a definite common substance or one clearly defined chemical group, it remains hypothetical so long as the assumed different elements have not been separated or established as individual structures by chemical methods (see 18). It could also be assumed that the phenomena are at least in part brought about by the action of one antibody on several antigens whose specific groups are similar but not identical (see 19, 4).

In order to examine the question raised, an attempt was made by specific precipitation to separate the carbohydrates into their hypothetical units. On the whole these experiments did not lead to obtaining fractions with different properties; and therefore the results did not support the idea of the existence of separable units in the single antigens. Indeed it would be desirable also to apply chemical methods for the purpose of fractionation of the specific carbohydrates.

A noteworthy difference between the observations reported and the results obtained with the carbohydrates of pneumococci by Heidelberger, Avery, and Goebel, is the following: The carbohydrates of the three fixed types of pneumococci exhibit very marked chemical differences in correspondence with their serological diversity. Such conspicuous chemical differences have not been found among the carbohydrate preparations from the Salmonella group, which, although serologically different, showed no very striking variation in sugar content and optical rotation, with perhaps one exception (*B. hog cholera*).

SUMMARY.

Specific precipitable substances rich in carbohydrates, containing very little protein and small amounts of a material apparently of fatty nature, have been prepared from the main serological types of the typhoid-paratyphoid groups. The preparations in their present

state of purity do not exhibit very pronounced chemical differences in spite of serological dissimilarity. In this respect the results differ from those observed with the polysaccharides of pneumococci.

The specificity of the precipitin reactions of these substances parallels in a general way the so called small flaking agglutination.

Attempts to separate different fractions from the active substance serologically by means of precipitation with antibody solutions were on the whole unsuccessful.

The differences in resistance to the action of acid and alkali were found to be characteristic for various specific carbohydrates.

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THE PRODUCTION OF PARTIAL LIVER INSUFFICIENCY IN RABBITS.

By PHILIP D. McMASTER, M.D., and D. R. DRURY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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Reasons enough for the existence of the liver are known. A host of functions have been ascribed to the organ already. Nevertheless the clinical condition of liver insufficiency is still a riddle. For the better understanding of it one must distinguish between functions which are essentially hepatic and those which can be carried out somewhere else in the body when the liver fails.

The recent work on dogs by Mann (1–9) and his colleagues has greatly increased our knowledge of liver functions. But much remains to be learned, and the method of these authors is laborious. For this reason we venture to submit a simple procedure for inducing extreme liver insufficiency in rabbits.

To approach the problem of liver insufficiency in animals three general types of procedure or their combination are available; total extirpation, partial ablation, and derangement of the organ by tying the bile duct or by chemical injury. The method of total extirpation, though it has served most excellently (1–9), still leaves much to be desired. Death follows the operation so rapidly that scant time remains for study of its onset. The use of drugs to bring about liver derangement is questionable for one must assume that the substance employed acts only on the liver parenchyma and not elsewhere as well. The evidence for and against these methods has been reviewed by Marshall and Rowntree (10) and by Mann (9).

The employment of partial ablation has also been open to objection. Investigators are agreed (9, 11, 12) that the liver possesses a "factor of safety" so great that it is well-nigh impossible to remove enough parenchyma to produce insufficiency without fatally obstructing the portal flow. Workers partially ablating the liver in dogs and rats

have found this difficulty insurmountable (9, 11, 13). In the rabbit our efforts have led to success. One can remove slightly more than 90 per cent of the animal's liver without untoward obstruction to the portal blood flow, with result in an extreme degree of hepatic insufficiency. The animals unless treated succumb in 12 to 18 hours, with convulsions and low blood sugar. If given glucose they live for varying periods up to 5 days, eventually dying with symptoms like those exhibited by dogs deprived of the entire liver (2, 9). For a study of the problems of liver insufficiency the method presents many advantages. Not the least of these is the ease of the ablation. It can be accomplished in less than 10 minutes, by a single operator, unassisted.

Anatomical Peculiarities of the Rabbit's Liver.

The arrangement of the rabbit liver in well-nigh separate lobes permits the removal of much of the hepatic substance by simple ligations and excision. Three large cephalad lobes, comprising what has been termed the "main liver" (14–16), constitute about 70 per cent of the organ. The remainder, almost separate from these, the "posterior lobe mass" (14, 15), is situated further from the head and to the right of the portal vein. Unlike the lobes of the "main liver" the "lobe mass" is intimately attached to the vena cava and cannot be wholly ablated without injury to this vessel. But a deep cleft exists on the cephalad surface of the mass and here a ligature may be thrown about the lobe and tied in such a way that it lies close to the vena cava but yet not close enough to impede the flow of blood. The half of the lobe beyond this ligature can now be removed, leaving a neat small stump. The small caudate lobe on the other side of the cava possesses a flattened, tail-like portion springing from a narrow base which can be readily severed after ligation. There remains about half the substance of the lobe, that portion which extends along the sheath of the portal vein between it and the spinal column.

In our attempts to induce liver insufficiency in the rabbit we employed three types of sub-total hepatectomy. Ablation of the "main liver" was the first. To this operation there was next added excision of the removable part of the caudate lobe, leaving the stump and the whole "posterior lobe mass" intact. Finally removal of the "main

liver" and half of the "posterior lobe mass" was practiced, leaving only the stump of this latter and the small caudate lobe intact. This last operation alone resulted in true liver insufficiency.

Estimation of the Relative Amounts of Liver Tissue Removed by the Three Types of Sub-Total Hepatectomy in the Rabbit.

In twenty-five normal rabbits we have separated the portions of the liver, discussed above, weighing the "main liver," the excisable portion of the "posterior lobe mass," its remaining stump, the removable bit of the caudate lobe, and its stump. Table I shows, in terms of percentage of total liver, the relative amounts of the tissue remaining and removed by each of the three procedures outlined above. Further, the table gives some idea of the relative variability in individual instances. Simple ablation of the "main liver" (see Column I of the table) withdraws on the average about 73.6 per cent of the liver tissue, a figure well in accord with the findings previously published by Rous and Larimore, 72.3 per cent (15), and by Ponfick, 74.7 per cent (16). As will be seen below, this procedure does not induce apparent liver insufficiency. "Main liver" removal plus partial ablation of the caudate lobe offers no great additional advantage over the former method, for the caudate lobe adds but little to the percentage of tissue removed (Column II). Ablation of the "main liver" and half the "posterior lobe mass" is the method of election (see Column III of the table). By this procedure an average of slightly over 90 per cent of the liver tissue is removed, the remaining stump of the "posterior lobe mass" and the intact caudate lobe constituting but 9.6 per cent of the original total.

Method.

Rabbits weighing 1500 to 4500 gm., previously kept on a mixed diet, were fasted but allowed water for periods of 24 to 48 hours. They were then operated upon under ether anesthesia. The three large cephalad lobes of the liver, the "main liver," were removed together with that portion of the "posterior lobe mass" which lies to the right of the vena cava.

To accomplish this, the "posterior lobe mass" of the liver was exposed and the ligament attaching its lower medial pole to the sheath of the vena cava severed. A heavy silk ligature was thrown around the lobe, placed in the deep incisura on its cephalad surface, and tied, care being taken to avoid all puckering of the vena cava or inclusion of its sheath in the knot. The liver portion to the right of the

TABLE I.

The Relative Amounts of Liver Removed by the Three Methods of Sub-Total Hepatectomy Described in the Text.

	No.	Body wt.	Liver percentage of body wt.	I "Main liver" out		II Posterior lobe in		III Caudate lobe in	
				Per cent of total liver		Per cent of total liver		Per cent of total liver	
				Out	In	Out	In	Out	In
		gm.							
Freshly fed	1	2570	2.32	76.8	23.2	78.6	21.4	93.4	6.6
	2	1960	2.70	72.7	27.3	76.1	23.9	90.8	9.2
	3	2150	2.21	68.1	31.9	72.2	27.8	90.7	9.3
	4	2957	2.41	76.5	23.5	81.2	18.8	90.2	9.8
	5	2250	2.88	81.2	18.8	83.9	16.1	89.4	10.6
	6	2010	2.22	76.2	23.8	81.0	19.0	89.1	10.9
	1a	4275	2.48	73.7	26.3	77.2	22.8	91.9	8.1
	1b	2375	2.36	80.1	19.9	84.2	15.8	91.2	8.8
Average.....		2568	2.45	75.7	24.3	79.3	20.7	90.8	9.2
Fasted 24 hrs.	1	1980	4.38	73.1	26.9	76.8	23.2	91.4	8.6
	2	2310	4.61	75.3	24.7	80.2	19.8	90.6	9.4
	3	2621	4.74	69.8	30.2	74.9	25.1	90.6	9.4
	4	2210	3.67	72.0	28.0	77.2	22.8	90.3	9.7
	x	1800	3.99	70.4	29.6	75.2	24.8	88.7	11.3
	y	2100	3.80					90.6	9.4
	z	2050	3.76					90.2	9.8
Average.....		2153	4.14	72.1	27.9	76.9	23.1	90.3	9.7
Fasted 48 hrs.	1	2250	3.17	75.2	24.8	78.4	21.6	92.4	7.6
	2	1770	2.53	69.8	30.2	73.6	26.4	90.1	9.9
	3	1450	3.12	66.8	33.2	71.8	28.2	89.5	10.5
	4	1960	3.20	68.1	31.9	73.7	26.3	88.1	11.9
	*	2150	2.25	76.8	23.2	82.2	17.8	88.3	11.7
Average.....		1916	2.85	71.3	28.7	75.9	24.1	89.7	10.3
Fasted 5 days	1	1509	2.83	75.9	24.1	78.1	21.9	92.1	7.9
	2	1500	2.87	74.2	25.8	77.9	22.1	91.3	8.7
	3	1550	3.02	74.3	25.7	80.3	19.7	90.3	9.7
	4	1600	2.63	72.6	27.4	75.5	24.5	89.5	10.5
	5	1750	2.75	72.8	27.2	76.6	23.4	89.1	10.9
Average.....		1582	2.82	74.0	26.0	77.7	22.3	90.5	9.5
Average of all.....				73.6	26.4	77.7	22.3	90.4	9.6

* Fasted 3 days.

ligature was then cut away. After severing the ligaments attaching the "main liver" to the diaphragm, a ligature was placed about it. This ligature, tied just ventral to the diaphragm without inclusion of the vena cava, permitted excision of the "main liver."

In some instances interference with the circulation of the remaining liver tissue resulted in necrosis of these parts. Rarely the vena cava was obstructed by the ligatures about the "posterior lobe mass" and very rarely a bile leak or hemorrhage occurred. Occasionally, too, animals developed a postoperative pneumonitis. Such instances have been ruled out in considering the evidences of liver insufficiency induced by this method. They have amounted in all to about 15 per cent.

After excision of nine-tenths of the liver tissue, in the rabbit the remaining portions appeared slightly congested but never turgid and tense as they do after the removal of but three-fourths of the organ in dogs and rats (11, 13). The venous channels became moderately dilated and a fatty infiltration of the parenchyma developed. Furthermore changes indicative of a fatty degeneration were found histologically, this serving to increase further the degree of liver insufficiency obtained by the operative procedure. Autopsy yielded no evidences of severe portal obstruction. There was no pronounced dilatation of the portal tributaries, and no marked congestion of the gastric or intestinal mucous membranes or engorgement of the spleen. Even the remaining portions of liver were not greatly distended with blood. Nevertheless we have asked ourselves, Can death have been due to obstruction to the flow of portal blood occasioned by the operation? To answer this question a series of control experiments were carried out in which an equivalent or greater obstruction of the portal blood flow was induced with but a negligible removal of liver parenchyma.

Control Experiments.

In seven rabbits, the portion of the "posterior lobe mass" of the liver lying to the right of the vena cava was removed under ether anesthesia as in the operation for nine-tenths liver ablation. The portal vessels to the "main liver" were then ligated, leaving intact all branches of the bile ducts and the hepatic artery. In this way the portal blood to 90 per cent of the liver was shunted through the caudate lobe and the remaining stump of the "posterior lobe mass." 4 to 8 days later, India ink was injected into the portal vein of the surviving rabbits to determine how great an amount of portal obstruction had actually been obtained. In

five instances the ink flowed only to the 10 per cent of liver which, according to the conditions of the experiment, should have received portal blood. In the two remaining rabbits some ink reached the main liver, showing that the portal blood had some access to it.

These animals in which a diversion of 90 per cent of the portal blood to the liver had been successfully effected had all survived in good condition.

In another group of four rabbits the branch of the portal vein which leads to the "posterior lobe mass" was ligated following ablation of the "main liver." This procedure caused an even greater blocking of the portal blood flow than that above described; yet the animals did not die until about 5 days after the operation. The sole road for the portal blood had been through the small caudate lobe. Marked hypertrophy of this lobe was found with dilatation of its channels and one may infer that obstruction to the portal flow had progressively diminished from the time of the operation. Death was preceded by the typical signs of liver insufficiency, to be outlined below.

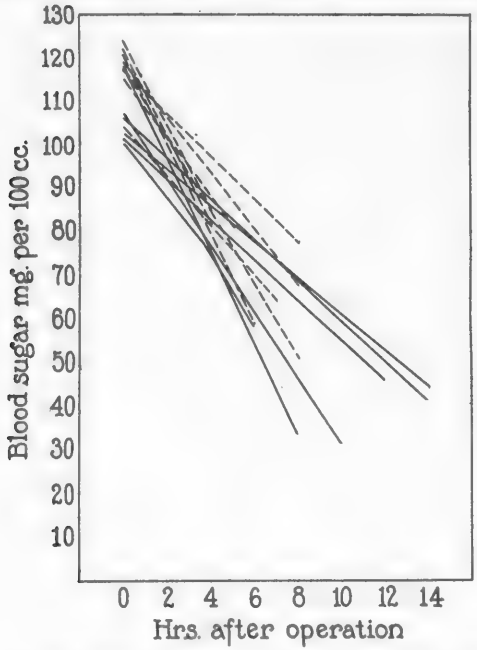
It is evident that death of rabbits when 90 per cent of the liver has been removed by our method cannot be ascribed to the portal obstruction incident to the operation. As will be reported in a succeeding paper, we have found it possible to occlude in the rabbit from 9/10 to 19/20 of the lumen of the portal vein together with 4/5 to 9/10 of the lumen of the vena cava without jeopardizing the animal.

The Evidences of Liver Insufficiency after Ablation of 90 Per Cent of the Rabbit Liver.

The Fall in Blood Sugar.—Following removal of nine-tenths of the liver the animals made a rapid recovery from the anesthetic, drank water, and appeared to be in excellent condition. In a few hours, however, they were to be found with drooping head, sprawling legs, and obvious muscular weakness. The heart rate was rapid, rising to 285 beats per minute from a preoperative average of about 140. In 6 to 12 hours the animals became prostrate and convulsions supervened, in one of which death occurred about 8 to 18 hours after operation. Mann and Magath have described (2, 3) a rapid fall in the blood sugar concentration of dogs deprived of the entire liver, with result in convulsions and death a few hours after the operation. So too in our rabbits deprived of 90 per cent of the organ the blood sugar fell rapidly to a level below the minimum compatible with life.

In numerous instances we have followed the blood sugar concentration in rabbits before and after removal of nine-tenths of the liver,

employing for the purpose the Hagedorn-Jensen method (18, 19). Text-fig. 1 shows the rapid decrease of blood sugar resulting from the liver loss, in fourteen of these animals. In the instances depicted by the solid lines, the blood sugar concentration fell to a level incompat-



TEXT-FIG. 1. *Fall in the Blood Sugar Concentration in Rabbits Following Ablation of 90 Per Cent of the Liver.*

The blood sugar findings in fourteen rabbits deprived of 90 per cent of the liver are plotted in terms of milligrams of glucose per 100 cc. of blood. In all the animals a rapid fall in the amount of blood sugar occurred. In the instances depicted by continuous lines, typical hypoglycemic symptoms developed and the animals died. The dotted lines show that a similar fall in blood sugar concentration took place in eight animals, until they were given glucose at the points where the lines are discontinued.

ible with life; convulsions and death followed. The dotted lines show an equally rapid initial fall in the blood sugar of other rabbits, which was checked by injections of glucose given at the points indicated by the discontinuance of the lines.

The Insufficiency in Rabbits Given Glucose after Ablation of 90 Per Cent of the Liver.

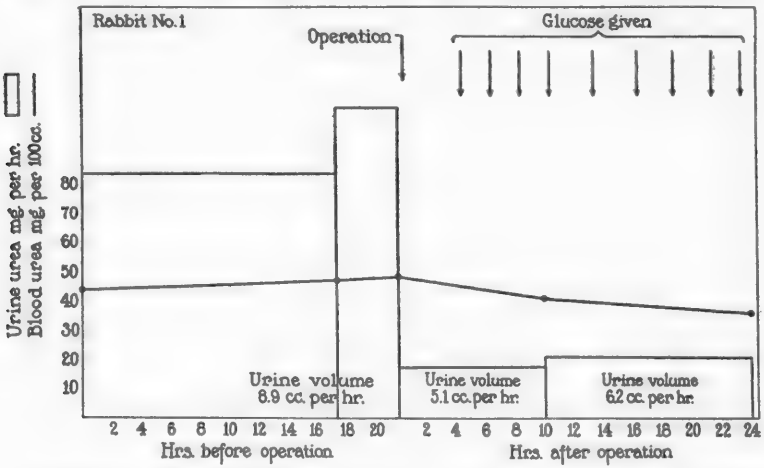
Mann and Magath showed (3) that administrations of glucose to dogs deprived of the total liver preserved life for many hours. Eventually the animals died, in spite of the maintenance of a high blood sugar level, in a condition characterized by weakness and flaccid paralysis. The same phenomenon more prolonged was found in our rabbits given glucose after ablations of about 90 per cent of the liver. The substance was usually administered by stomach tube, but often by intravenous injection, and at times subcutaneously, in varying doses and at various intervals of time.

The effect of glucose, especially by intravenous injection, in rabbits deprived of 90 per cent of the liver is as startling as in completely hepatectomized dogs (3, 9). Prostrated, moribund animals will be found sitting up or moving about freely a few minutes after a single injection of 10 to 20 cc. of 5 or 10 per cent dextrose solution. The pulse and respiratory rates are slowed and the animal appears normal again. The effect, however, is not long lived. 30 minutes to several hours later the rabbit again becomes weak, the pulse and the respiration rate are increased, the blood sugar is low, reflexes are exaggerated, and convulsions may occur. Again and again the animal may be restored by administrations of glucose but finally, in spite of these, death comes on, heralded by extreme asthenia, coma, and respiratory failure.

Derangements in Urea and Uric Acid Metabolism.—It is now well known that urea formation ceases in the liverless dog (20). This phenomenon is accompanied by an accumulation of uric acid in the blood, with a consequent increased excretion of the substance if kidney function is maintained. The change from a normal urea metabolism does not appear in dogs deprived of as much as 70 per cent of the liver after an Eck fistula operation (9, 21), although uric acid destruction may possibly fail to occur. Indications of a lack of urea formation or of cessation of uric acid destruction in dogs may be taken as definite evidence of liver insufficiency (9). Such evidence has been obtained in our partially hepatectomized rabbits.

Procedure.—Freshly fed rabbits were fasted for 24 hours, given 50 to 75 cc. of 5 per cent glucose solution by gavage, and later allowed to drink the same solution

at will. A copious secretion of urine resulted. 12 to 20 hours later the animals were catheterized, the bladder washed thoroughly with water, and a blood sample taken from an ear vein. They were then placed in clean metabolism cages for 17 to 24 hours. At the end of this period another blood specimen was taken, and the catheterization and bladder washing repeated. The urine thus obtained and that collected during the previous 17 to 24 hour period was analyzed for urea and uric acid. At once after taking the second catheter specimen 90 per cent of the liver was removed under ether anesthesia, and for varying periods thereafter

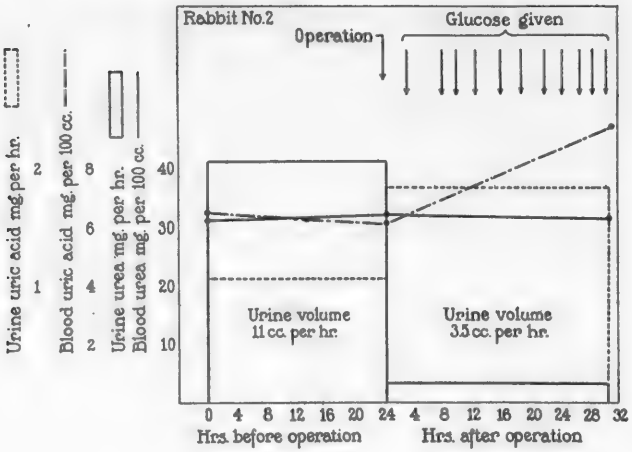


TEXT-FIG. 2. *Changes in Blood Urea Concentration and Urea Output in the Urine after Removal of 90 Per Cent of the Liver.*

Uric acid studies were not made. The changes shown are similar to those described in Text-figs. 3 to 6.

the animals were kept in metabolism cages. From time to time they were given water or 5 per cent glucose solution to maintain diuresis. At intervals they were catheterized and the urine and bladder washings added to the cage urine specimens to be analyzed for uric acid and urea. Blood specimens taken during or immediately before the catheterizations were used for blood urea and blood uric acid analyses. For the determinations of urea concentration in blood and urine, the method described by Addis (22) was employed. In some instances (Text-figs. 2, 4, and 5) the technique of Van Slyke (23) was used as well. Folin's method (24) served for the estimation of uric acid in the blood, and the procedure of Folin and Wu (25) for its determination in urine.

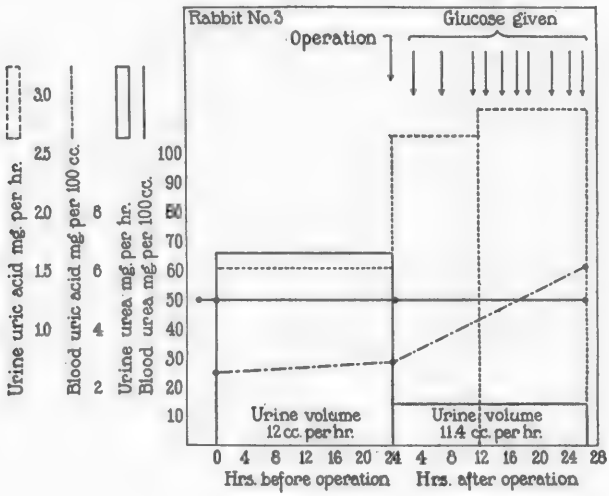
In all five instances depicted in Text-figs. 2 to 6 adequate diuresis was maintained. In all a striking decrease of urea output in the urine


Rabbit No.2
Operation
Glucose given
Urine uric acid mg. per hr.
Blood uric acid mg. per 100 cc.
Urine urea mg. per hr.
Blood urea mg. per 100 cc.
Urine volume 11 cc. per hr.
Urine volume 3.5 cc. per hr.
Hrs. before operation
Hrs. after operation

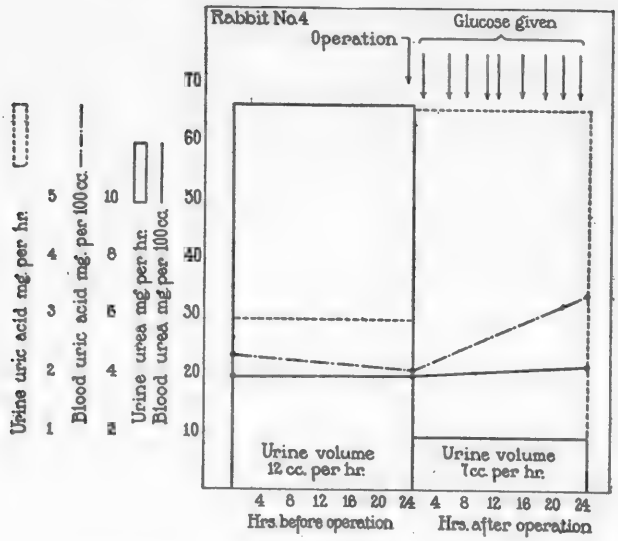
TEXT-FIG. 3.

TEXT-FIGS 3 to 6. *Changes in the Urinary Output and Blood Concentration of Urea and Uric Acid in Rabbits Deprived of 90 Per Cent of the Liver.*

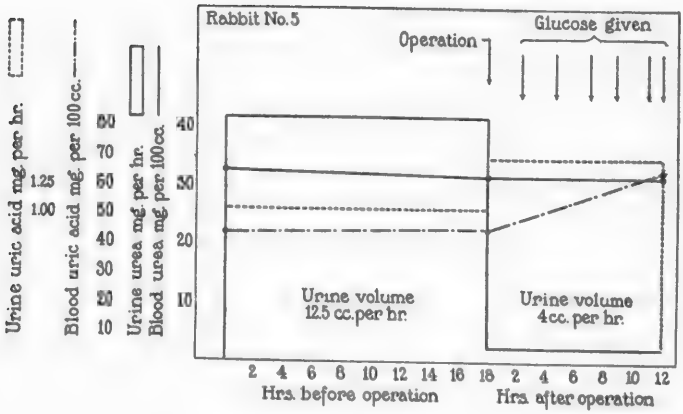
Text-figs 3 to 6 depict the uric acid and urea findings in the blood and urine of rabbits before and after ablation of 90 per cent of the liver. In all, the urea output of the urine decreased profoundly after the ablation while the blood urea concentration remained almost unchanged. In all, an increased urinary output of uric acid appeared, together with a greater concentration of the substance in the blood.


Rabbit No.3
Operation
Glucose given
Urine uric acid mg. per hr.
Blood uric acid mg. per 100 cc.
Urine urea mg. per hr.
Blood urea mg. per 100 cc.
Urine volume 12 cc. per hr.
Urine volume 11.4 cc. per hr.
Hrs. before operation
Hrs. after operation

TEXT-FIG. 4.


Rabbit No.4
Operation
Glucose given
Urine uric acid mg. per hr.
Blood uric acid mg. per 100cc.
Urine urea mg. per hr.
Blood urea mg. per 100cc.
Urine volume 12 cc. per hr.
Urine volume 1cc. per hr.
Hrs. before operation
Hrs. after operation

TEXT-FIG. 5.


Rabbit No.5
Operation
Glucose given
Urine uric acid mg. per hr.
Blood uric acid mg. per 100cc.
Urine urea mg. per hr.
Blood urea mg. per 100cc.
Urine volume 12.5 cc. per hr.
Urine volume 4cc. per hr.
Hrs. before operation
Hrs. after operation

TEXT-FIG. 6.

occurred. Despite this lack of urea output the blood urea concentration remained unchanged in three of the animals, fell in one (Text-fig. 2), and rose but slightly in two, affording thereby evidence of greatly decreased urea formation. The uric acid studies made on four of these animals, Text-figs. 3 to 6, showed an increase of this substance in both blood and urine after ablation of 90 per cent of the liver.

In considering the evidences of liver insufficiency in these animals it is to be noted too that a mild tissue icterus appeared, accompanied by an output of bile pigment in the urine, as shown by the Van den Bergh test.

Liver Insufficiency in Rabbits Deprived of 80 Per Cent of the Liver.

Similar studies were made on three animals deprived of but 80 per cent of the liver. The findings were not clear-cut. The almost complete absence of urea excretion observed after ablation of 90 per cent of the liver was not present in these instances. In two of the animals the urinary output of urea per hour amounted to about half that of the preoperative period, while in the third animal no decrease was observed. Despite this the blood urea concentration of all rose notably, yielding evidence of a normal formation of urea in the case of the animal last mentioned, and of only a slight decrease in the others. In but one of the three experiments was an increase in urinary uric acid noted and this appeared in the instance showing normal urea formation. From these findings one might suppose that by removal of 80 per cent of the organ a borderline condition had been obtained in which there might or might not be liver insufficiency, in respect to these functions.

The Degree of Liver Insufficiency in Rabbits Following Ablation of 70 Per Cent of the Organ.

Prior to the adoption of the method described above, the "main livers" of several rabbits were ablated in the hope that the animals would develop a true liver insufficiency, for the operation entails a loss of 70 per cent of the organ. In 1889 Ponfick (16) reported his results with this procedure, stating that practically all the animals died. Of twenty-one rabbits operated upon in our series, seven survived in

good health for several weeks, when they were autopsied. Two others died of postoperative pneumonia and five others lived about 6 days, succumbing from undetermined causes. In the remaining seven instances, death occurred in about 20 hours accompanied by very low blood sugar concentration and convulsions. Ordinarily one finds after removal of the rabbit's "main liver" a transient fall in the blood sugar which may be sufficient to bring about death. The other evidences of liver insufficiency, the clinical picture of asthenia and prostration with faulty urea and uric acid metabolism, are in our experience lacking.

DISCUSSION.

The evidence presented herein clearly shows that a fatal liver insufficiency develops in rabbits deprived of 90 per cent of the organ. Following this great reduction in parenchyma the blood sugar falls to the lethal point. If this be avoided by the administration of dextrose the insufficiency manifests itself in a partial failure of the organ to form urea and to transform uric acid. Eventually the animal dies, of causes unknown.

After ablation of 90 per cent of the liver a mild jaundice appears, showing that there is a deficiency in the function of bile elimination. Earlier work from this laboratory, on the biliary obstruction required to produce jaundice in dogs, is in agreement with this finding. McMaster and Rous showed that jaundice of biliary obstruction does not appear after ligating several of the branches of the hepatic duct unless the drainage from 95 per cent or more of the liver is obstructed (12).

The fact is important that the rabbit deprived of 90 per cent of its liver behaves like the liverless dog in all ways that have thus far been tested. The blood sugar concentration falls, convulsions occur, urea formation ceases, uric acid metabolism is disturbed, and even when the blood sugar is artificially maintained at a high level death occurs, with symptoms similar to those arising in the liverless dog (9).

SUMMARY.

A rapid and simple method for the production of marked liver insufficiency in rabbits has been described. The necessary operation can be carried out by an unassisted operator in a few minutes. The method should further the study of liver physiology.

The changes as concerns blood sugar, urea formation, and uric acid metabolism would appear to be the same in the rabbit suffering from hepatic insufficiency as in the dog.

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TOTAL SURGICAL REMOVAL OF THE LIVER IN RABBITS.

By D. R. DRURY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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Total surgical removal of the liver in the rabbit without impairment of the portal and caval circulation has not hitherto been reported. A method has been devised for the purpose of work in this laboratory. It would seem to be desirable to report its technique not only because of the wide use of the rabbit as an experimental animal but because of the frequency with which it has been employed in studies upon the relation of the liver to carbohydrate metabolism (1).

Excision of the liver necessitates occlusion of the portal vein. Moreover the intimate attachment of the organ to the vena cava requires the removal of a segment of this vessel as well, if a complete and satisfactory liver ablation is to be obtained. In the normal rabbit the obstruction of either, or both, of these veins is followed by death so rapidly that in a method for hepatectomy channels must be provided for the immediate return of caval and portal blood to the heart. Markowitz and Soskin (2) have reported a technique for inducing a collateral circulation in dogs without an Eck fistula. At a preliminary operation the portal vein and vena cava are partially occluded with ligatures which constrict the lumina of these vessels to about one-fifth the original size. After some weeks collaterals to the veins are so well developed that the liver may be removed without impairment of the portal circulation. The present method is a modification of this technique.

Our first attempts with the rabbit were unsuccessful. The vena cava and portal vein had been approached directly, and exposed on the right side of the abdomen. The consequent injury to the ventral surface of the liver and the peritoneum of this region resulted in massive adhesions among the injured parts, preventing removal of the liver without great blood loss. It therefore became imperative to devise a method whereby these veins could be approached, and

partially ligated, from the left side of the abdomen, without exposing the liver, leaving the right side free of adhesions that would hinder the later hepatectomy.

Anatomical Peculiarities of the Rabbit's Liver.

The liver of the rabbit consists of two relatively separate masses connected by a thin isthmus of parenchyma. The larger mass, comprising the three cephalad lobes, has been termed the "main liver" and the smaller portion, the "posterior lobe mass" (3). Each possesses its own arteries, ducts and branches of the portal vein. The main portal trunk on approaching the liver divides to send its first branch to the "lobe mass." At about this level it also receives a tributary—the superior pancreatico-duodenal vein—and in this region too the hepatic artery comes to lie close to the vessel and accompany it to the liver. Still another tributary, the left gastro-epiploic, enters the portal vein about 1 cm. cephalad to the juncture of the portal and superior pancreatico-duodenal veins. The relationship of these structures is by no means constant. Thus, for example, the superior pancreatico-duodenal vein may enter the portal stream either below or above the great branch of the portal to the "posterior lobe mass" of the liver. As the aim of the preliminary operation is the establishment of a portal obstruction so great that it will induce the development of a collateral circulation, it is necessary to produce this obstruction caudad to the first branch of the portal vein, that to the 'lobe mass." Unfortunately the tributaries mentioned above, the superior pancreatico-duodenal and the left gastro-epiploic, usually enter the portal vein above this site of election and, as our experience has shown, will serve as by-passes, whereby portal blood reaches the "main liver." To cut off this source of supply it is necessary to ligate the two small vessels individually.

The Preliminary Operation.

The shaved skin of the rabbit is swabbed with 60 per cent alcohol and under ether anesthesia the abdominal cavity is exposed from the level of the ensiform to the umbilicus by an incision 1 cm. to the left of the mid-line. The relatively bloodless mid-region is thus left intact for the incision of the secondary operation. The stomach is pressed upward, covering the liver, and the portal vein is approached from the left side and sufficiently freed of the surrounding tissue for a

silk ligature, soaked in petrolatum, to be passed about it. The ligature is placed just caudad to the branch to the "posterior lobe mass" and to the junction of the superior pancreatico-duodenal and portal veins. Great care should be exercised to place the ligature around the vein only, leaving intact between it and the liver the peritoneum adherent to its right side, thus preventing all contact between silk and the liver. Only if this is successfully accomplished will adhesions fail to develop.

The ligature is tied down so as to include not only the vein but the end of a glass rod. This end is bent at a right angle to the main shaft and is laid parallel to the vessel for ease in tying. It should taper slightly so that it can be readily withdrawn after the tie has been accomplished leaving the vein partially ligated, with a lumen equal to the size of the rod. The diameter of the latter should be 2 mm. for a 2 kilo animal.

The superior pancreatico-duodenal vein is tied near its junction with the portal, and likewise the small vein which arises on the caudad surface of the pylorus and leads into the portal about 1 cm. above the entrance of the superior pancreatico-duodenal vein. At times this vessel may enter the portal vein just dorsal to the pylorus. Both these procedures, carried out without injury to the peritoneum of the right side of the abdomen, are essential; for as already mentioned these veins, unless occluded, enlarge with extreme rapidity and soon provide an adequate supply of blood to the liver. The ligation of the small left gastro-epiploic vein can be carried out only by a dissection along the posterior wall of the pylorus. The vein receives, as a rule, one or more tributaries. It should be inspected along its entire course and the ligature placed below the last tributary, a delicate procedure which exposes the bile duct and portal vein in this region as well.

Finally partial occlusion of the vena cava is effected from the left side of the abdomen. As a first step the superior mesenteric artery is identified and the peritoneum perforated on the left side of the mesentery just cephalad to this artery and just ventral to the aorta. A small glass spatula covered with vaseline is introduced into the aperture and worked between the vessel and the peritoneum anterior to it. A threaded ligature passer introduced along the track thus made is turned posteriorly about the vena cava, thus carrying a ligature around the vessel, just caudad to the right adrenal body. The lumen of the vena cava is then reduced to 2 mm. by tying the ligature about the removable glass rod as in the case of the portal vein.

Recovery from the operation is usually rapid and a collateral circulation soon develops about the liver. In a few days, if the procedure has been successful, evidences of this fact appear, in a notable enlargement of the veins of the abdominal wall. The development of this collateral circulation is so rapid that one can totally occlude the portal vein and the vena cava 5 days after the primary operation without the appearance of any untoward symptoms. This has been done in 4 instances as a control experiment to demonstrate the existence of an adequate by-pass for the blood about the liver. In our later work, however, we have gener-

ally allowed an interval of 3 weeks to elapse between the primary and secondary operations. This not only affords ample time for the development of the new vascular bed about the liver but suffices for the animal's complete return to general health. Liver extirpations have been done successfully as late as 6 months after the preliminary operation.

Hepatectomy in the Rabbit.

The incision for hepatectomy in the rabbit should run in the mid-line from ensiform to umbilicus. A ligature is placed around the entire gastrohepatic omentum, including in the one tie the portal vein, bile duct and hepatic artery. The vena cava is ligated just cephalad to the right adrenal gland, and again just cephalad to its union with the hepatic veins from the "main liver," and severed between. The structures in the gastrohepatic omentum are cut cephalad to the tie placed about them. The liver can now be removed *in toto*, after cutting its ligaments to the diaphragm and dividing the peritoneum on either side of the vena cava in the regions where this is intimately attached to the liver. No stump of hepatic tissue need be left.

A diaphragmatic vein enters the right side of the vena cava slightly caudad to the entrance of the hepatic veins from the "main liver." This small vessel must be tied and cut between ligatures.

In certain instances it may be of advantage to remove the "main liver" early in the operation, immediately after tying the gastrohepatic omentum, thereby gaining working space for the more difficult steps. This is done by snipping the ligamentous attachments between the "main liver" and diaphragm and throwing a stout ligature around the former, tying it down closely upon the vena cava. The "main liver" is then cut away above this ligature. In the final stage of the operation, its stump is removed.

DISCUSSION.

The clinical picture in the rabbit, deprived of the entire liver, needs only brief mention for in all important particulars it is like that in the liverless dog, described by Mann (4). As is now well known, hepatectomy in the dog is followed by pronounced hypoglycemia with an accompanying train of typical symptoms. Further, this condition fails to appear when the diminution of blood sugar is prevented by administrations of glucose. The postoperative state in the liverless dog may be divided into two clearly defined stages, the first characterized by the hypoglycemic symptoms, the second, even if glucose be given, by coma terminating in death.

All these phenomena have their counterpart in the rabbit, as our observations upon these animals deprived of 90 per cent of the liver

(5) have already shown. Completely hepatectomized animals treated by the administration of dextrose in 25 per cent solution *per os* or in 5.4 per cent solution intravenously in sufficient amounts to keep the blood sugar concentration at or about 125 mg. per cent behave normally for from 12 to 32 hours. In this first stage no untoward symptoms appear. As a rule only slight variations of the pulse and respiratory rate are noticed, together with an increase in body temperature of 1 to 1.5°. Finally, in spite of the glucose administrations, certain phenomena usher in what may be called the second stage. Now the animal when placed on the floor no longer investigates its surroundings in the ordinary way but hops blindly in a straight line, striking any object in its path, even a brilliantly lighted wall. Later on evidences of muscular weakness and ataxia appear; the animal sits with sprawling legs and drooping head. Left in the cage it will be found with nose pressed in a corner. In the next few hours, although care is taken that the blood sugar level does not fall below normal, the weakness progresses and the movements become more ataxic. The animal falls to one side in attempting to move and cannot rise again, or it lies with sprawling legs unable to raise its head or draw the limbs under the body. The temperature becomes subnormal, the respiratory movements are labored and less frequent, and the pulse is slower too. The corneal reflex and knee jerk previously present are lost and complete flaccid paralysis, save of the respiratory muscles, sets in. For a period Cheyne-Stokes respiration may occur, after which the respiratory rate falls to 8 per minute or even lower, and while the pulse rate remains at about 30 per minute the heart sounds progressively weaken. After varying periods up to 40 hours death comes on, with respiratory failure, the heart continuing to beat for 10 minutes or more after respiration has ceased.

At the present time we can venture no explanation of the later symptoms. It is of importance now merely to call attention to the close similarity of the postoperative events in the liverless rabbit and dog, a similarity which permits us to infer perhaps that the major functions of the organ are much the same though the one creature is herbivorous and the other omnivorous.

SUMMARY.

A technique is described for total removal of the liver of the rabbit without circulatory difficulties as a result. The method requires a preliminary operation to induce a development of portal and caval collaterals.

Rabbits deprived of the liver in this manner if given glucose live for varying periods up to 40 hours. Before death they show the same disturbances as do hepatectomized dogs. They die early, of hypoglycemia, unless provided with sugar.

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THE RELATION OF THE LIVER TO FAT METABOLISM.*

I. Effect of Liver Lack on Fat Combustion and the Respiratory Quotient.

By D. R. DRURY, M.D., and PHILIP D. McMASTER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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It is generally agreed that the liver is active in fat metabolism in many ways. As early as 1886 this became evident to Nasse (1). Subsequent systematic studies by Noel Paton (2), Leathes (3, 4), Leathes and Raper (5), Raper (6), Mottram (7), Rosenfeld (8), Bloor (9, 10, 11) and others, have resulted in a mass of evidence, demonstrating various liver activities in the metabolism of fat. But are these functions performed solely by the liver and are they vital to the organism? Can fat combustion be carried on at all in the absence of the organ? These are the chief questions concerning the rôle of the liver in fat metabolism. Their answer waits upon a demonstration of the absence or continued presence of fat combustion in animals deprived of the liver or otherwise brought into a state of extreme hepatic insufficiency. In the present communication we will report experiments upon this theme. Rabbits were chosen for the work and total liver ablations done (12) by a method already described. For the induction of a partial but pronounced liver insufficiency, removal of approximately 90 per cent of the liver was practiced (13).

The Respiratory Quotient of Rabbits with Partial Liver Insufficiency.

Earlier workers have shown that the respiratory quotient of fasting animals, previously well fed, is low (14), indicating a body metabolism largely that of fat. What now will the quotient be after removal of the liver, or in conditions of hepatic insufficiency?

* A preliminary report upon some of the findings given herein has already appeared in the *Proceedings of the Society for Experimental Biology and Medicine*, 1927, xxv, 151.

Well nourished rabbits on a mixed diet, and weighing 2 to 3 kilos, were fasted 48 to 72 hours and subjected to a preliminary determination of the respiratory quotient by the method outlined below. The respiratory quotient was low in such fasting animals indicating a pronounced combustion of fat. Then liver insufficiency was induced.

Method.

The Respiratory Quotient. The Apparatus.—All respiratory quotient determinations were made by the closed circuit method with the rabbits sealed in an air tight respiration chamber, a very large desiccator, submerged in a constant temperature bath. The chamber was just large enough to allow the animals the normal crouching posture. The closed circuit consisted of the animal respiration chamber, two parallel absorbing systems with shut-off clamps, a mercury pump which afforded a continuous circulation of air, and a set of valves permitting the air to pass through the circuit in one direction only. The total volume of the apparatus was 11 liters. The absorbing systems, for the removal of water vapor and CO_2, were each made up of an ascarite[1] tube connected at both ends with sulfuric acid absorption bottles. The weight of the ascarite tube in one of these systems was carefully determined, and so too was that of the second sulfuric acid bottle which the air entered after passing through the ascarite. The circulating air could be shunted at will through either of these absorption circuits. From a reservoir of known volume, oxygen entered the closed circuit between the absorbers and the pump. At this point too, connections were made with an auxiliary oxygen reservoir. At frequent intervals the apparatus was tested for leaks, by raising the pressure by an amount equal to that of 100 cm. of water in addition to the atmospheric pressure and ascertaining that no volume change occurred in the circuit within 5 minutes.

The Respiratory Quotient Determination.—The respiration chamber containing the experimental animal was submerged in the constant temperature bath and the air within the system circulated at the rate of 3 liters a minute. But one of the absorption systems was employed in the circuit and oxygen was used from the auxiliary oxygen reservoir. After 20 minutes the pump was stopped and the pressure within the system adjusted to that of the room atmosphere. The barometric pressure was noted at the same time. Upon starting the circulation of air again the current was deflected through the second train of absorbers containing the weighed ascarite tube and weighed sulfuric acid adsorption bottles. Oxygen was now allowed to enter the closed system from the measured reservoir until about 1.5 liters had been consumed. This required about 1 hour and 20 minutes with the average 2 to 2.5 kilo rabbit. At the end of this period the pump was

[1] "Ascarite," a mixture of asbestos and sodium hydroxide, distributed by A. H. Thomas Company, Philadelphia.

stopped, the pressure in the closed system adjusted again to that of the room atmosphere, the barometric pressure observed, and the volume of oxygen added to the system during the period was noted. By reweighing the ascarite tube and second sulfuric acid bottle the amount of CO_2 produced was determined. The volume of oxygen added during the period of the experiment, plus or minus the correction for changes in barometric pressure, temperature and vapor pressure, represented the oxygen consumed.

The rabbits remained quiet within the respiration chamber. It was not necessary to prevent all motion on the animal's part for calorimetry studies were not contemplated and only the gas exchange was measured. However the animals sat so tranquilly that we were able, in later work, to give them continuous intravenous injections of glucose while in the chamber, despite the fact that the injecting needle once placed in an ear vein could not be readjusted during the period of the respiratory quotient determination. In over 20 such experiments no animal ever moved sufficiently to dislodge the needle from the vein. The method will be described further on.

Technique.—When it had been established that the respiratory quotient of a fasted rabbit was definitely low, indicating that fat combustion was taking place, blood specimens were taken from an ear vein for sugar, hemoglobin and hematocrit estimations, and immediately thereafter 90 per cent of the liver was ablated under ether. 5 to 7 hours later, when the immediate effects of operation and anesthesia had worn off, a second determination of the respiratory quotient was made. Immediately preceding the animal's entrance into the respiration chamber and at once following its removal therefrom, samples of venous blood were taken by cardiopuncture from the right ventricle for CO_2 and sugar analyses.

As we have shown in a preceding paper the blood sugar concentration falls rapidly after removal of 90 per cent of the liver, reaching the lethal minimum 6 to 10 hours after the operation. As the second respiratory quotient determinations were made in the latter part of this period the blood sugar level was invariably found low, between 60 and 70 mg. per cent. Often the animals collapsed and became moribund while in the respiration chamber the second time. In such instances the experiments were discontinued.

In four instances a third respiratory quotient was determined on the following day, 24 hours or more after the removal of 90 per cent of the liver. These animals, of course, received glucose during the night in amounts just sufficient to keep the blood sugar level slightly above the minimum required to keep them free from convulsions. Blood sugar analyses were made before the final respiratory quotient was taken to rule out instances in which the giving of too much glucose might have brought the blood sugar concentration to normal or above. It seemed conceivable in this event, indeed it has been shown by our later work, that the utilization of glucose by the animal might mask or entirely supplant the combustion of fat. In one instance, Table I, No. 4, the blood sugar concentration was found high (0.099 per cent) on the day following operation and the respiratory quotient determination was duly postponed several hours, until it had fallen to 0.079 per cent.

TABLE I.

Respiratory Quotient, Metabolic Rate and Blood Sugar Findings in Rabbits Deprived of 90.6 Per Cent of the Liver.*

No.	Preoperative findings			6 to 8 hours after operation			24 hours after operation		
	Respiratory quotient	O_2 consumption	Blood sugar	Respiratory quotient	O_2 consumption	Blood sugar	Respiratory quotient	O_2 consumption	Blood sugar
		mg. per min.	*mg. per cent*		*mg. per min.*	*mg. per cent*		*mg. per min.*	*mg. per cent*
1	0.740	27.5	0.114	0.720	27.8	0.064	—	—	—
2	0.766	20.7	0.119	0.757	18.5	0.069	—	—	—
3	0.783	19.3	0.118	0.720	18.9	0.077	—	—	—
4	0.729	28.0	0.122	—	—	—	0.731	32.6	0.079
5	0.782	33.6	0.128	—	—	—	0.745	31.9	0.056
6	0.768	30.0	0.125	—	—	—	0.735	29.5	0.046
7	0.760	25.5	0.122	0.739	25.0	0.059	0.743	26.1	0.066
8	0.826	29.9	0.128	0.751	27.7	0.085	0.714†	29.6	0.051

* Expressed in terms of O_2 consumption per minute.

† 10 hours after operation.

TABLE II.

Blood CO_2 of Rabbits Immediately before and after the Period of Respiratory Quotient Determination.

No.	Before entering respiration chamber	After entering respiration chamber	Hours after operation
	vol. per cent	*vol. per cent*	
1	48.1	48.3	6
2	46.8	47.6	6
3	39.6	39.9	6
4	47.5	47.2	24
5	40.1	40.5	24
6	42.7	43.1	26
7	44.4	44.4	5
7	41.6	40.6	24
8	46.0	45.6	6
8	45.8	45.5	10

As an additional routine measure, blood specimens from the ear vein were taken before operation and at variable intervals thereafter for sugar estimation by the Hagedorn-Jensen (15) method. After operation, blood specimens for CO_2 analysis were taken immediately preceding, and at once following the sojourn of

the animals in the respiration chamber. They were obtained by cardiopuncture of the right ventricle, under oil, and in paraffined tubes. The CO_2 estimations were carried out by the method of Van Slyke and Sendroy (16, 17). These latter findings for the instances shown in Table I are presented separately in Table II. As the respiration chamber was small and the animals quiet, differences in the metabolic rate could be roughly determined in terms of oxygen consumption per minute.

Only those instances have been considered in which the metabolic rate remained constant before and after operation (see Table I). All experiments have been ruled out, too, in which any significant change in the concentration of blood CO_2 was found. The results given below must be attributed to the true gas exchange of the animal and not to mere retention or blowing off of CO_2 during the periods of experimentation.

In these experiments, which were of relatively brief duration, no significant hemoglobin or hematocrit changes were observed.

Findings.

The preliminary respiratory quotients of 25 fasted rabbits averaged 0.755, with variations between 0.722 and 0.826, indicating a great combustion of fat. In Table I the respiratory quotient, blood sugar and oxygen consumption data are given in 8 of these instances which constitute experiments free from objection, in which neither blood CO_2 changes nor significant variations in the metabolic rate appeared. The magnitude and character of the individual variations are self-evident. When about 90 per cent of the liver of the animals was ablated, the respiratory quotient 6 to 8 hours later averaged 0.737, with individual variations between 0.720 and 0.757. The respiratory quotients of 4 of these rabbits taken again the following day averaged 0.738, with only slight variations,—0.731 to 0.745.

In 4 other successful experiments, not shown in the table, the respiratory quotient and blood sugar findings were similar but the metabolic rate showed some retardation, the animals requiring $1\frac{3}{4}$ to 2 hours, even $2\frac{1}{2}$ hours in one instance, to consume the quantity of oxygen used prior to operation in about $1\frac{1}{4}$ to $1\frac{1}{2}$ hours. These latter instances are merely corroborative and hence need be mentioned no further.

From the findings reported so far we conclude that fat combustion

can be carried on as readily and as rapidly in the rabbit after ablation of 90 per cent of the liver as before, in spite of the fact that the animals suffer from extreme liver insufficiency, eventually dying therefrom (13).

The Respiratory Quotient of Rabbits Deprived of the Entire Liver.

With these facts established, it became essential to determine whether fat combustion could continue in the absence of the entire liver. In the experiments just described, there was the possibility that the findings depended upon some activity of the small remnant of the liver. For this reason we repeated the experiments, as described above, but employed rabbits deprived of the entire organ according to the method described in an earlier paper (12).

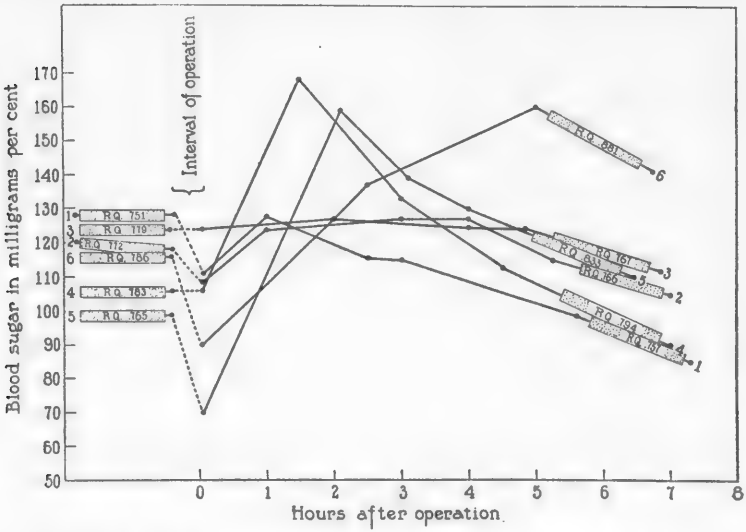
One important change in technique was forced upon us. The rabbits deprived of the entire liver required glucose prior to the postoperative estimation of the respiratory quotient. For the fall in blood sugar concentration took place more rapidly in them than in those animals retaining 10 per cent of the organ. In the liverless rabbit given no glucose death from low blood sugar occurs in some instances within 2 to 3 hours after operation; and it seemed wise to wait at least 4 hours before determinations of the respiratory quotient since both metabolism and respiratory quotient might have been disturbed by the ether anesthesia and laparotomy. Preliminary experiments were made to determine the minimum amount of glucose which would maintain hepatectomized rabbits. These experiments will be detailed in a following communication. Suffice it to say here that 100 to 130 mg. of glucose per kilo of body weight per hour answers the purpose, when given by continuous injection during the first 8 hours after hepatectomy. This small amount of glucose only was given in the later work, since a larger dosage might conceivably have acted to mask an existing fat consumption.

Technique.

For the respiratory quotient experiments a technique was used similar to that just described, with certain minor modifications.

Rabbits of about 2 kilos were subjected to subtotal ligations of the portal vein and vena cava to establish a collateral circulation about the liver (12). 3 to 5 weeks later the animals were fasted for 72 hours and given water which they drank freely. The long fasting period was found necessary, for the respiratory quotient of a previously well nourished rabbit, which is fasted, does not fall in less than 3 days. At the end of this time if a preliminary respiratory quotient determination was found to be low, as it usually was, blood specimens were taken for sugar estimations and hepatectomy done under ether anesthesia.

At once after operation blood sugar determinations were again made and a continuous intravenous injection, of isotonic (5.4 per cent) glucose solution, begun. The change in the blood sugar percentage found at this second estimation deter-



TEXT-FIG. 1. *The Blood Sugar Concentration of Rabbits Deprived of the Entire Liver and Given Small Amounts of Glucose during the Interval between the Preoperative and Postoperative Respiratory Quotient Determinations.*

The blocked in areas indicate the period during which respiratory quotient determinations were made and their height above the base line the blood sugar levels. Blood sugar estimations were done after each of the preoperative respiratory quotient determinations and their level plotted accordingly. In each instance a blood sugar estimation was made again immediately after the operation for hepatectomy. The changes in concentration are shown by the dotted lines. At various intervals thereafter the blood sugar concentration was determined in each animal and the variations depicted by the continuous lines. As the blood sugar levels were known both before and after the postoperative respiratory quotient determinations were made, the chart shows the approximate level of blood sugar concentration during those periods. It is obvious that the blood sugar concentration fell during the postoperative respiratory quotient determinations.

mined how much glucose was later given, the amount being that deemed necessary just to maintain the blood sugar concentration above the level of 100 mg. per cent. Experience soon taught us that it was safer to give too much glucose than

too little, for in two experiments the animals entered the state of hypoglycemic collapse while in the respiration chamber before the respiratory quotient determinations could be made. These experiments, of course, were ruled out. Three of the instances plotted in Text-fig. 1 received slightly too much glucose and a distinct rise in the blood sugar level followed. At various intervals blood sugar determinations were made as guides to the rate at which glucose was injected later.

After the operation, during the continuous glucose injection, the animals sat quietly on a warmed pad, with only occasional shifting movements.

About 5 to 6 hours after removal of the liver a second estimation of the respiratory quotient was begun; and in one instance a third was made 24 hours after hepatectomy. Ordinarily the animals remained in the respiratory quotient chamber about 1½ hours. The glucose injection was continued during this period, the fluid entering the chamber through a glass tube connected in turn with fine rubber tubing about 8 inches long, to which the needle was attached. The needle inserted in the ear vein was kept in place by two weak bulldog clips and the rubber tube supported along the animal's back by an adhesive strip to prevent its occlusion by a kink should the rabbit shift its position. In all of the experiments the glucose injection was successfully carried out.

To determine changes in the blood CO_2 and sugar, specimens of venous blood were taken in every instance, immediately before and again after the animal's sojourn in the respiration chamber. To obtain blood samples in these experiments cardiopuncture was not necessary since the animals presented greatly enlarged collateral abdominal veins from which blood was taken, under oil, in paraffined syringes.

As in the previous experiments, we shall consider only the cases in which the metabolic rate remained high after operation and no significant blood CO_2 changes could be demonstrated.

Findings.

It was deemed sufficient to carry on experiments with hepatectomized rabbits until 6 instances had been obtained which fulfilled the conditions mentioned above. Table III gives a survey of the findings with the 6 different animals, on one of which (No. 4) a third respiratory quotient was obtained 24 hours after operation. In Text-fig. 1, we have charted the blood sugar curves of these animals in such a way as to show the *probable* blood sugar level during the period of respiratory quotient determinations.

Of the 6 instances, 4 showed practically no change in the respiratory quotient after liver removal, although in 1 of these (No. 4) a late determination was made as long as 24 hours after the operation. In

TABLE III.

The Respiratory Quotient and Metabolic Rate in Rabbits Deprived of the Entire Liver.*

The respiratory quotients of 6 fasted rabbits before and after removal of the liver are shown, together with the rate of oxygen consumption during each respiratory quotient determination. After hepatectomy all these animals received glucose. In Columns 4 and 9 the "noncarbohydrate" respiratory quotient has been calculated as explained in the text.

No.	Preoperative		Postoperative							
	Preliminary respiratory quotient	O_2 consumption	Second respiratory quotient	"Noncarbohydrate" respiratory quotient	Hours after operation	O_2 consumption	Third respiratory quotient	"Noncarbohydrate" respiratory quotient	Hours after operation	O_2 consumption
	A	B	C	D	E	F	G	H	I	J
		mg. per min.				*mg. per min.*				*mg. per min.*
1	0.751	17.22	0.757	0.715	6	16.40	0.862†	—	8†	15.91
2	0.772	20.53	0.766	0.749‡	5½	19.35	—	—	—	—
3	0.779	18.03	0.767	0.709	5½	17.08	—	—	—	—
4	0.783	19.84	0.794	0.729	5½	20.18	0.786	0.715	24	18.84
5	0.765	17.82	0.833§	0.765	5	15.48	—	—	—	—
6	0.786	21.12	0.881§	0.748	5½	16.92	—	—	—	—

* Expressed in terms of O_2 consumption per minute.

† Excess of glucose given as described in text.

‡ Did not receive enough glucose during respiratory quotient determination.

§ Received too much glucose before and during respiratory quotient determination. Blood sugar high.

TABLE IV.

Blood CO_2 of Rabbits Deprived of Total Liver Immediately before and after the Period of Respiratory Quotient Determination.

No.	Before entering respiration chamber	After entering respiration chamber	Hours after operation
	vol. per cent	*vol. per cent*	
1	43.35	43.14	6
2	39.35	38.98	5½
3	40.18	39.95	5½
4	41.63	41.34	5½
4	44.80	45.40	24
5	37.16	37.58	5
6	45.60	45.42	5½

2 instances an appreciable rise in the respiratory quotient occurred. It may here be remarked again that of necessity all the animals received continuous intravenous glucose injections during the interval between the operation and the completion of the respiratory quotient estimation.

In both of the animals in which a rise in the respiratory quotient occurred, Nos. 5 and 6, a marked fall in blood sugar was noticed immediately after the liver ablation (see Text-fig. 1). To prevent the development of hypoglycemic symptoms, much more glucose was given to these animals than to the others. In Rabbit 6, far too much glucose was given, 200 mg. per kilo per hour, and the blood sugar level stood at 159 mg. per cent at the beginning of the respiratory quotient determination. The other instance showing a rise in the respiratory quotient (No. 5) also received much more glucose per kilo per hour than those in which no rise was found. As Text-fig. 1 shows, the blood sugar concentration in this instance was abnormally low after operation. The animal was consequently given 185 mg. of glucose per kilo per hour instead of the usual 100 to 130 mg. Although the blood sugar concentration in the animal was not high immediately before the period of respiratory quotient estimation, it is to be noted that the animal had received this excess sugar.

The findings show beyond doubt that fat combustion continues actively in the animal without a liver.

The Effect of Increased Sugar Administration on the Respiratory Quotient of Hepatectomized Rabbits.

During the respiratory quotient determinations, the period that is to say when the blood sugar level could not be ascertained, the amount of glucose given was arbitrarily cut down to avoid the production of a sugar combustion sufficient to mask any existing one of fat. On the assumption that this glucose, given during the periods of respiratory quotient estimation, was burned we have calculated the hypothetical "non-carbohydrate-burning respiratory quotients," shown in Columns D and H of Table III. These approach closely in some cases the figure 0.71, that of a wholly fat burning respiratory quotient. As Text-fig. 1 shows, the blood sugar concentration of each animal was lower at the end of the respiratory quotient determination than before,

a finding which would support the assumption that the sugar introduced had been used up.

In calculating the hypothetical "non-carbohydrate-burning respiratory quotient" of Columns D and H in Table III we are for the moment assuming that the administration of glucose raised the respiratory quotient. To test the point one experiment was done.

In the experiment on Rabbit 1 a respiratory quotient of 0.757 was obtained between the 6th and 7th hours after operation. For 6 hours prior to this the animal had been given 100 mg. of glucose per kilo per hour by continuous intravenous injection. The blood sugar level fell from 126 mg. per cent 1 hour after liver removal to 0.098 just before the respiratory quotient determination. During the period of the respiratory quotient estimation the glucose dosage was further decreased to 80 mg. per kilo per hour, causing a further fall in the blood sugar level to 0.086, as Text-fig. 1 shows.

The animal was then given an intravenous injection of 5 cc. 5.4 per cent glucose and the rate of the continuous injection increased to the level of 300 mg. per kilo per hour. 20 minutes later another respiratory quotient determination was begun. This required 1½ hours, during which glucose was given at the rate of 300 mg. per kilo per hour. The quotient rose to 0.862. To avoid confusion with the findings in the other instance the latter part of the experiment has been omitted from Text-fig. 1.

DISCUSSION.

It is not to be inferred from our findings that the respiratory quotient must be low under all conditions after removal of the entire liver or a 90 per cent fraction of it. In this paper we wish to emphasize merely that the organism deprived of the liver is still capable of burning fat adequately for its needs. An existing fat combustion may be easily masked, as shown by our experiments in which too much glucose was administered, purposely or inadvertently to the liverless animals. And the respiratory quotient may be high in a variety of circumstances after hepatectomy. Thus, for example, if it is high before operation, it will tend to remain so thereafter. One of our "unsuccessful" experiments serves to illustrate this point.

Ablations of 90 per cent of the liver were carried out on two rabbits which had been fasted but 36 hours. This was done following the preliminary respiratory quotient estimations but before the results had been calculated. At operation the stomachs of the animals were found moderately distended with food. The preliminary respiratory quotients were 0.865 and 0.851, respectively. In both

instances, the respiratory quotient was found high 6 to 8 hours after operation, 0.811 and 0.826, respectively. Autopsy disclosed quantities of undigested food in the stomachs, sufficient reason for the high quotients. In another experiment, a freshly fed rabbit on a carbohydrate diet was subjected to the same procedure. The respiratory quotient which was close to unity (0.951) remained at this figure 6 hours later.

In another rabbit with a preliminary respiratory quotient of 0.731 the second determination was purposely taken 2 hours after operation and found to be 0.840. Guided by this finding we considered it advisable as routine to wait several hours after hepatectomy before attempting the second respiratory quotient estimations.

From the evidence given here one can only say with certainty that fat combustion continues unaffected after removal of the liver of animals already burning fat before operation. The state in which they are burning fat can be brought about by fasting, for about 3 days, animals previously well fed. The freshly fed rabbit on a carbohydrate diet shows as a rule a high respiratory quotient, and so does one fasted for less than 48 hours. On the other hand the fasting period should not be unduly prolonged. For one would of course expect a respiratory quotient indicative of protein combustion in an animal suffering from severe inanition.

Some experimenters have in the past employed the respiratory quotient as an indicator of the type of metabolism existing in animals after attempted exclusion of the liver from the general circulation by the ligation of vessels. In 1910 Porges (18) and Porges and Solomon (19) found a rise in the respiratory quotient of rabbits and depancreatized dogs following ligation of the abdominal aorta, the inferior vena cava, the portal vein and the hepatic veins. They concluded that the oxidation of fat and protein proceeds within the body only in the presence of the liver. In these experiments the animals lived but a few hours, rapidly becoming moribund. Rolly (20), too, reported changes in the respiratory quotient after interferences with the circulation of the liver but found them inconstant. Verzar (21, 22) obtained inconstant results after partial exclusion of the liver by anastomosis of the portal vein with the inferior vena cava. Grafe and Fischler (23) reported no change in the respiratory quotient of dogs with Eck fistulas. Fischler and Grafe (24) ligated the hepatic artery in Eck fistula dogs and observed usually a rise in the respiratory quotient within 6 hours of the operation. But it is to be noted that the respiratory quotient returned to the preoperative figure in the animals surviving more than 6 hours. Bohm (25) excluded the abdominal organs in depancreatized dogs and found but little rise in the respiratory quotient. Still later Grafe and Denecke (26) extirpated the liver in dogs several weeks after an Eck fistula had been formed and found the respiratory quotient low—in one instance 0.774 after operation.

Several explanations for these varying results have since come to hand. As is now well known, from the work of Mann (27) and others, an evident liver lack can only be brought about by almost complete removal or destruction of the organ. Rich (28) has recently demonstrated the futility of attempted exclusion of the liver from the circulation through the methods practiced by most of the earlier workers. Murlin, Edelmann and Kramer (29) have pointed out that radical ligations, excluding as they do much blood and tissue, should be carefully controlled. These authors have demonstrated that changes in the relationships of the blood gases follow sudden clamping of the abdominal aorta which are of themselves sufficient to account for the results of the earlier workers without the need to invoke, as explanation, a change in the metabolism of the animals.

Although no radical circulatory interference, such as complicated these various findings, has been brought about in our experiments we have thought best to rule out the possibility of errors due to changes in the blood CO_2 and have done so by considering only instances in which no variations of the sort occurred.

Mann (27, 30) has reported a series of carefully conducted studies upon the respiratory quotient of hepatectomized dogs. In his experiments the respiratory quotient tended to approach unity after removal of the liver. That the quotient in dogs need not necessarily be close to unity after hepatectomy is shown by the work of Markowitz (31).[2] From our findings on rabbits and the figures given in his paper we believe that the respiratory quotient of hepatectomized dogs would be found low 6 hours after the operation if fat combustion were duly established before liver removal and the giving of glucose afterwards reduced to a minimum.

SUMMARY.

Fat combustion is carried on adequately in rabbits deprived of the liver or brought into a condition of extreme liver insufficiency. Even 24 hours after hepatectomy fat combustion goes on as well as in the normal animal. Evidently the liver plays no essential part in the breaking down of fat.

[2] Following the completion of this work, F. C. Mann and W. M. Boothby reported experiments in the *American Journal of Physiology*, 1928, lxxxvii, 486, which show an increase in the respiratory quotient of dogs immediately after removal of the liver. A subsequent return of the respiratory quotient 17 hours later to a figure but slightly above the preoperative one was also observed.

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ON THE MECHANISM OF OPSONIN AND BACTERIOTROPIN ACTION.

I. Correlation between Changes in Bacterial Surface Properties and in Phagocytosis Caused by Sera of Animals under Immunization.

By STUART MUDD, M.D., BALDUIN LUCKÉ, M.D., MORTON McCUTCHEON, M.D., and MAX STRUMIA, M.D.

(*From the Henry Phipps Institute and the Department of Pathology, University of Pennsylvania, Philadelphia.*)

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The opposing views of the early cellular and humoral schools of immunologists were to some degree brought into harmony by the demonstration by Denys and Leclef (1), Wright and Douglas (2) and others that phagocytosis could be greatly increased by the presence of serum. Components of normal sera promoting phagocytosis only in relatively high concentration were called by Wright and Douglas *opsonins*. The specific components of immune serum, active either in high or low concentration, have been termed *bacteriotropins* (3). It was shown that the action of opsonins and bacteriotropins was chiefly upon the bacteria or other cells phagocytized, not upon the leucocytes. Treatment by appropriate sera prepared bacteria or other cells for phagocytosis even after the removal of the serum.

Study of the phagocytosis of particulate matter brought out the fact that a number of factors in the phagocytic system could influence the degree of phagocytosis. Thus phagocytosis of carbon particles suspended in sodium chloride solution could be increased somewhat by addition of small amounts of calcium chloride, ethyl alcohol or iodoform, and decreased by departure of the suspending sodium chloride solution from isotonicity (4). Manganese dioxide particles showed an attraction for leucocytes and were very rapidly taken up by them; no such attraction could be demonstrated for carbon or quartz particles (5). Effects on the activities of delicate living cells like leucocytes by factors in their environment are not surprising. However, such effects are not comparable to the enormous increases in phagocytosis produced by the action of bacteriotropins on the cells to be phagocytized.

Important progress was made by formulation of the interfacial tension relations in phagocytosis through the work of Rhumbler (6), Tait (7), and especially of Fenn (8). Fenn showed that in a given phagocytic system the amount of phagocytosis was proportional to the number of collisions between leucocytes and

particles (9). The chance that any given collision may result in phagocytosis would seem to depend to an important degree upon the *interfacial tension relations* at the colliding surfaces; these must be such that the leucocyte and particle will adhere and that the former will spread around and engulf the latter (8). It is understood, however, that the consistency of the protoplasm (10, 11, 12) of the leucocyte and possibly other factors may also enter into the phenomenon.

If then the surface properties of cells are of great importance in determining phagocytosis, have sera any such effects on cell surfaces as might account for their opsonic and bacteriotropic efficiency? Much evidence has been accumulating of late years to indicate that sera do profoundly change the surfaces of cells with which they interact. Studies on bacteria (13), erythrocytes (14), and spermatozoa (15), using the method of cataphoresis, and on erythrocytes (16) and acid-fast bacteria (17), using a method dependent on interfacial tension, have shown that all of these cells do, indeed, undergo certain characteristic changes in surface properties with serum sensitization. The present experiments indicate that these changes are intimately related to phagocytosis.

The experiments described in this paper and the one immediately following represent the first stage of a study directed toward analysis of the mechanism of opsonin and bacteriotropin action in physical-chemical terms. They are intended to answer the question, "What changes do sera effect in acid-fast bacteria in preparing them for phagocytosis?"

Methods.

Various strains of acid-fast bacteria, treated with serial dilutions of normal and immune sera, have been studied as follows:—(1) The bacteria remained in serum dilutions overnight and the *agglutination* readings were then made for each tube. (2) The serum-bacterial mixtures were strongly centrifugated and the sediments were *resuspended* by shaking until the untreated control tubes showed even suspension. (3) The sensitized bacteria were washed and their wetting or interfacial tension properties were then estimated in the *interface reaction*. The bacteria were observed microscopically in an oil-water boundary surface. (4) The cataphoretic velocity of the washed, sensitized bacteria was measured in a microcataphoresis cell. These 4 reactions together gave a picture of the cohesiveness, of the wetting properties, and of the surface potential difference of the bacteria tested for phagocytosis. (5) While such data were being obtained mixtures of rabbit leucocytes with, (a) bacteria and serum dilutions, or, (b)

sensitized, washed bacteria were rotated in stoppered vials on a Robertson agitator (18). Smears were made from each mixture, fixed and stained, and 100 (or 200) leucocytes in each smear were observed microscopically. The percentage of leucocytes which had taken up bacteria was recorded.

Such details of the several reactions as seem necessary are given below:

Bacteria were removed with a platinum loop from the surface of a glycerol-agar slant. They were rubbed against the bottom of a Pyrex test tube by a glass rod with rounded end. A few drops of 0.85 per cent sodium chloride solution were added after the first grinding, and the bacterial mass was rubbed up into a paste; this was diluted with 0.85 per cent sodium chloride with further grinding and shaking. This suspension was largely freed from clumps by centrifugation and brought to the desired turbidity with saline.

1. Agglutination.—1 cc. of the bacterial suspension was mixed with 1 cc. of each serum dilution. The tubes were left in the ice-box over night and in the morning were usually removed to the 37° room for 30 to 60 minutes before reading. Agglutination or sedimentation was read without shaking up the sediment. ++++ indicates complete agglutination with clearing, +++, ++, + and tr. indicate decreasing degrees of agglutination or sedimentation. The lesser degrees of agglutination recorded in many experiments with mammalian tubercle bacilli may well have indicated only sedimentation and not macroscopic agglutination, strictly speaking. Such sedimentation was never considered positive however, unless distinctly in excess of the sedimentation in the saline control tube.

2. Resuspension.—Under various special conditions, for instance with mammalian tubercle bacilli, agglutination as ordinarily carried out is unsatisfactory. To meet these cases a modified agglutination or "resuspension" reaction has been developed (19). After making the agglutination readings, all tubes were centrifugated at high speed until the bacteria were completely sedimented or practically so. The supernatant fluid was decanted and two drops of 0.85 per cent sodium chloride solution were added to the bacterial sediment in each test tube. The tubes were arranged in a rack with the control tubes in the middle, *i.e.*, tubes in which the bacteria had been mixed with 0.85 per cent sodium chloride solution without serum. The rack was now shaken uniformly until the sediment in the control tubes was first brought into even suspension. The bacteria which had been sensitized resuspended in flocculi whose coarseness increased with the concentration of serum and with the affinity of the serum components for the particular bacteria used.

This reaction has the advantage of eliminating certain imperfectly controlled variables in the second or flocculation stage of the agglutination reaction. The bacteria after sensitization are forcibly pressed together by centrifugal force.

Their subsequent resuspension depends primarily upon and gives a roughly quantitative estimate of their cohesion.

3. Interface Reaction.—After resuspension, 0.85 per cent sodium chloride solution was poured into each tube, all tubes were again centrifugated, the supernatant liquid was decanted, and the sediments were again resuspended. A portion of this sensitized washed suspension in each tube was used for the interface reaction (17) and a portion for cataphoresis. In the present experiments, the bacteria were observed microscopically in the interface between tricaprylin and 0.85 per cent sodium chloride solution. The degrees of alteration of the wetting properties of the bacteria after sensitization are charted as plus signs.

4. Cataphoresis.—A slightly modified form of the Northrop-Kunitz (20) microcataphoresis cell was used. For details see (21). The cataphoresis cell was mounted over a dark-field condensor. Three readings were made at each of 3 levels, namely, at $\frac{1}{12}$, $\frac{5}{12}$, and $\frac{8}{12}$ of the distance from the bottom to the top of the inside of the cell. A better technique used in the later experiments was to make 3 readings at each of the 2 "stationary levels," namely, at $\frac{83}{100}$ and at $\frac{70}{100}$ of the inside depth of the cell (22). The algebraic sum of the rates of migration at the several levels was used for calculation. The cataphoretic velocity is given in μ per second per volt per centimeter fall in potential along the cataphoresis cell. If we make certain assumptions (23), which are not rigidly accurate, however, velocities may be converted into millivolts potential difference at the bacterial surface by multiplication by 12.6.

Phagocytosis.—The importance of using serial dilutions of sera in careful phagocytosis work has long since been demonstrated (3, 24), but has been neglected. Moreover much work in phagocytosis has been vitiated and discredited by the difficulties and uncertainties of the Leishman-Wright opsonic index method. Introduction of the beautiful technique of Robertson and his co-workers (18) has therefore marked an important advance. The Robertson apparatus has been adapted to the needs of the present experiments, and a technique for staining and handling several titration series at the same time has been developed.

Leucocytes.—Exudative leucocytes from rabbits were used as the phagocytic cells according to a method elaborated in Hamburger's laboratory (25). They were obtained by injecting about 200 cc. of sterile 0.9 per cent sodium chloride intraperitoneally (on the right side about the mid-clavicular line and midway between costal margin and pelvis) into a rabbit by means of a hollow needle attached to a rubber tube and funnel; after 3 to 4 hours about 80 to 100 cc. of the fluid was recovered by puncturing the peritoneal cavity with a stout (No. 15 bore) needle near the mouth of which a few side openings had been drilled. The fluid was received in a flask containing a solution of 0.7 per cent sodium chloride + 1.1 per cent sodium citrate in proportion of 3 parts of peritoneal fluid to 1 part of saline-citrate solution. (In order to obtain fluid very rich in leucocytes an injection of 200 cc. of sterile 0.9 per cent sodium chloride solution was given on the evening before the experiment, to be followed in the morning by a second injection as described above.) The suspension was centrifuged for 2 minutes at a speed

of 1100 revolutions per minute; the centrifuge was started and stopped gradually; the supernatant fluid was poured off and the sediment resuspended in 0.9 per cent sodium chloride and centrifuged again for 2 minutes at the same speed. About 0.1 cc. of leucocytes were obtained from 15 cc. of peritoneal fluid. Usually the leucocytes from 3 rabbits were used, furnishing about 10 cc. of a very dense leucocytic suspension. Approximately 95 per cent of the cells were polymorphonuclear leucocytes; disintegrated cells and large monocytes constituted the remaining 5 per cent. The rabbits showed no ill-effects from the injection and withdrawal of fluid. The same rabbits were used weekly for 8 months. When leucocytes were obtained oftener a different series of rabbits was employed.

Phagocytic Mixture.—Into a series of small hard-glass vials of uniform size (50 mm. in length, 9 mm. inside diameter), 0.1 cc. of serum dilutions was placed (a fresh pipette was used for each dilution to insure greater accuracy). Immediately before adding 0.2 cc. of the leucocytic suspension (which was never more than ½ hour old), 0.1 cc. of bacterial suspension was introduced. Each of the vials contained, therefore, 0.4 cc. of fluid. They were stoppered with freshly paraffined well-fitting corks, and immediately placed on a Robertson rotating machine. The speed of rotation was 4 times per minute; the rotation was continued for 15 minutes. Temperature was that of the room, 25°±1.5°C. At the end of this period the tubes were quickly placed in racks and plunged in ice water for several minutes. A minute drop of human serum (heated for 30 minutes to 56°) was added to each vial immediately before making the spreads; this caused the leucocytes to adhere to the slide. The first vial of each series did not require the addition of serum.

Making of Spreads.—Spreads were made on thoroughly cleaned and polished numbered glass-slides. A small drop of the mixture was placed near one end of the slide with a clean pipette, using a fresh pipette for each vial, and drawn across the slide by a spreader (a slide with the corners broken off); the edge of the spreader was then dipped into a beaker of water and wiped clean and dry. The spread slide was rapidly dried by holding it before an electric fan which was standing in front of a radiator or electric heater. Rapid drying is essential for obtaining well-spread non-contracted leucocytes. The dried slides were exposed for 2 to 10 minutes to formalin vapors (by placing them, spread side downwards) over flat dishes containing undiluted formalin. After removal from formalin vapors they were placed in a row and the flame of a Bunsen burner passed rapidly over them.

Staining.—The fixed spreads were stood in staining racks and placed for 10 minutes in a covered jar containing Ziehl-Neelsen carbol-fuchsin previously heated to 70°C. They were then thoroughly rinsed in water. Each slide was decolorized separately by shaking it, with forceps, for 5 to 10 seconds in 10 per cent sulfuric acid. (This was found to decolorize the cells satisfactorily; the bacilli were brilliantly stained.) The slides were counterstained, usually for 20 seconds, in a 1:10,000 Azure II solution; (the dilution was made from a more concentrated solution; the diluting distilled water was rendered slightly alkaline by adding 0.3

cc. of a 15 per cent sodium carbonate solution to each 200 cc. of water). The staining solution was always freshly prepared. The slides were then rapidly washed, and dried, first between blotting papers and then by holding them in front of an electric fan (or placing them in the incubator).

Estimation of Degree of Phagocytosis.[1]—The numbers of leucocytes that had ingested bacilli were determined by counting 100 cells (sometimes 200 cells). Each of two observers counted 50 (or 100) cells; the results agreed very closely. Care was taken not to regard mere adherence to the leucocyte as evidence of phagocytosis. In the vast majority of instances there was no doubt as to whether a cell had ingested bacteria or not.

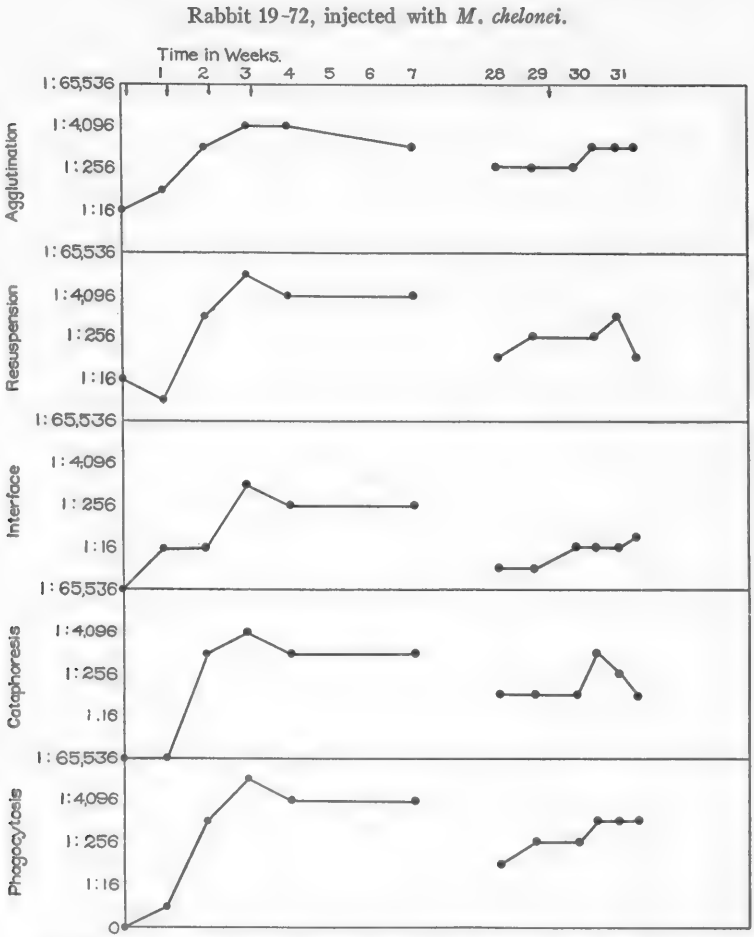
The agglutination, resuspension, interface and cataphoresis reactions were done at the Phipps Institute by one group of workers; portions of the sera and bacterial suspensions used in these tests were meanwhile sent to the other collaborators at the Medical School, by whom the phagocytosis tests were made. The results were not combined until both sets of observations had been completed.

By use of the reactions and procedure outlined above we are able to follow changes in the cohesiveness, the surface potential difference and the wetting properties of the bacterial surfaces, and to compare these changes with the increase in phagocytosis brought about by the same sera on other portions of the same bacterial suspensions. Positive resuspension and interface reactions each indicate increased cohesiveness between the bacteria of the suspension tested. A positive interface reaction indicates also bacterial surfaces less readily wet by oil than are those of the controls. The change (decrease) in surface potential difference is directly proportional to the decrease in cataphoretic velocity brought about by serum sensitization. Each of these changes in bacterial surface properties is closely correlated with the increase in phagocytosis brought about by serum, as will be shown in this and the two succeeding papers.

In Text-figs. 1 to 4 are plotted the titers for the several reactions of the sera of 4 rabbits, as withdrawn during the course of active immunization. The times in weeks between the beginning of the experiment and the bleedings of the rabbits are plotted on the abscissa axis.[2]

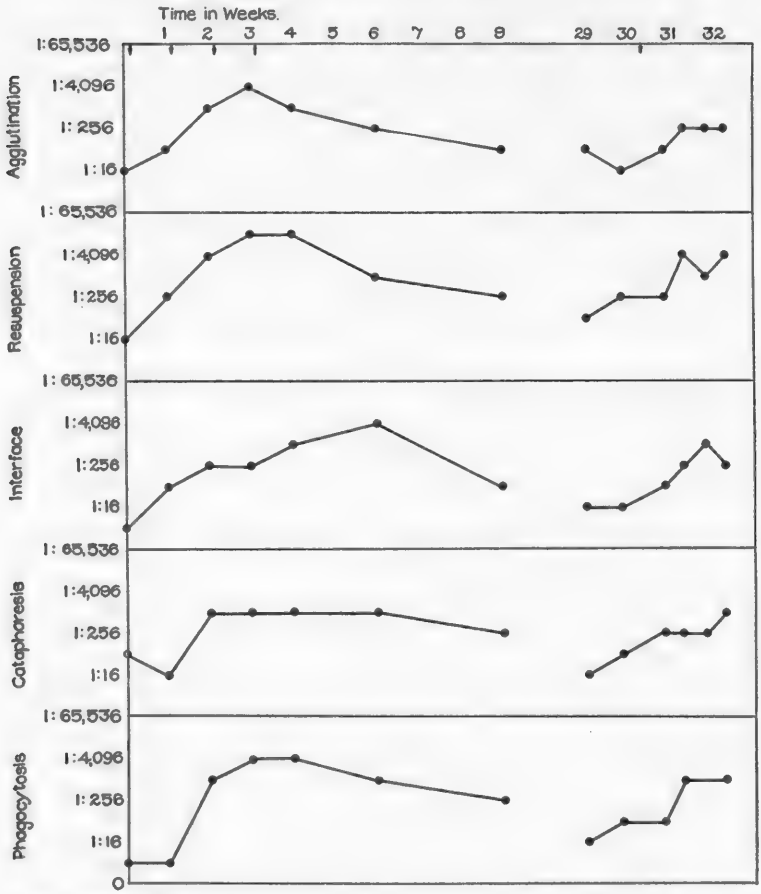
[1] In the present study phagocytosis is defined as ingestion of bacilli by polymorphonuclear leucocytes; the effect on the bacilli ingested is not here under consideration, nor is the question of phagocytosis by other types of cells than polymorphonuclear leucocytes.

[2] The sera were separated from the freshly clotted blood, and were inactivated for 30 minutes at 56°C. before use. The saline bacterial suspensions were ad-



TEXT-FIG. 1. The course of active immunization of a rabbit with turtle bacillus. Titers of the several reactions are plotted against time in weeks. Time of intravenous injections of living antigen is indicated by arrows. Note the parallelism between phagocytosis and bacterial surface changes in this and succeeding text-figures.

Rabbit 20–02 injected with *M. avium* (Arloing strain).

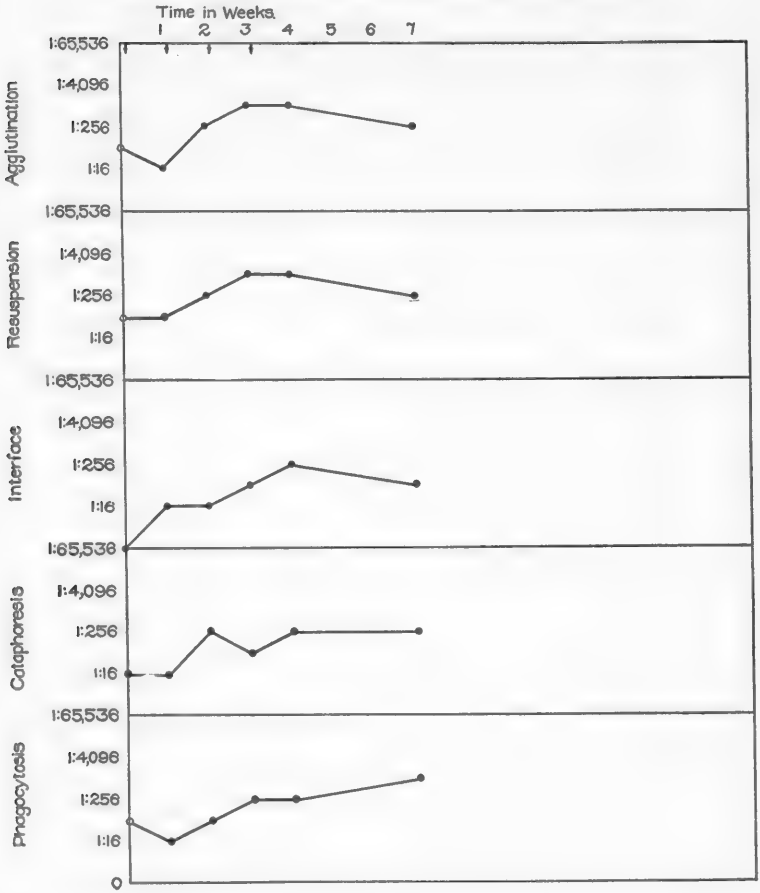


TEXT-FIG. 2. Active immunization of a rabbit with an avian tubercle bacillus. The open circle (first cataphoresis titer) indicates titer not reached but estimated.

Intravenous injections of saline suspensions of the living bacilli used for immunization are indicated by small arrows on this axis. The

justed as nearly as possible by comparison with a turbidity standard to the same turbidity for each experiment; the living microorganism homologous with the antiserum was used for all tests.

Rabbit 20–03, injected with *M. avium* (Prague strain).

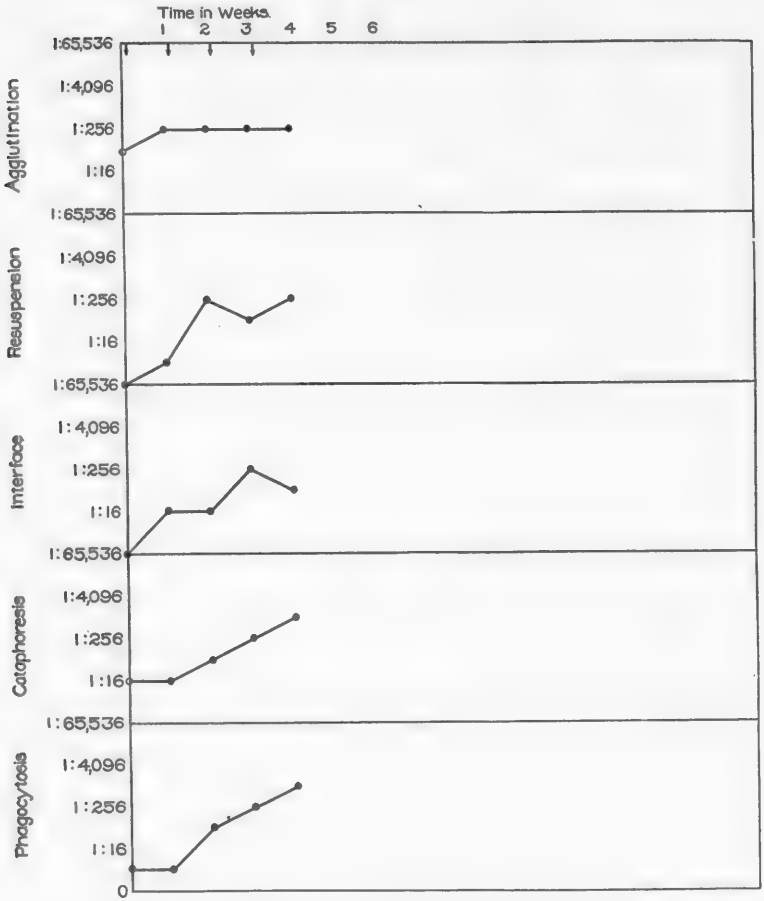


TEXT-FIG. 3. Active immunization of a rabbit with an avian tubercle bacillus. Symbols as in Text-figs. 1 and 2.

titers of the sera are indicated on the ordinate axis; the highest dilutions of the sera giving clearly positive results are taken as titers; traces or doubtful reactions are not included.

Text-figs. 1 and 2 show the course of active immunization due to

Rabbit 20–37, injected with *M. tuberculosis* (Bovine III strain).



TEXT-FIG. 4. Active immunization of a rabbit with an avirulent bovine tubercle bacillus. Symbols as in Text-figs. 1 and 2.

4 weekly injections, the subsequent slight falling of titer, the titers after a 5 months interval and the rise following a final injection of the antigens. The rabbits yielding the data shown in Text-figs. 3 and 4 died of intercurrent disease after the first immunization.

The general correspondence is very striking between the bacterial surface changes and the increase in phagocytosis brought about by the sera at all the stages of immunization. The absolute titers for the several reactions do not necessarily correspond; this was to have been anticipated, however, since it had already been recorded, for instance, that the interface reaction was relatively insensitive (17), and that agglutination with mammalian tubercle bacilli (see Text-fig. 4) was unsatisfactory (19). An occasional point also is obviously off the correct curve (*e.g.*, Text-fig. 2, interface value at 6 weeks). However the significant fact is the striking general parallelism of the titers indicating bacterial surface change and phagocytic increase; this parallelism is, we believe, complete, within the limits of experimental error.

Influence of the Intrinsic Properties of the Bacterial Surface.—Serum which prepares bacteria for phagocytosis, then, has in approximately corresponding degree altered the bacterial surface properties, *i.e.*, increased the cohesiveness, decreased the surface potential difference and altered the wetting properties. The correspondence between these bacteriotropic and surface effects strongly suggests that they are due to a common cause, namely, to deposition of a sensitizing substance or substances from the serum on the bacterial surface. It has already been shown that serum agglutination involves a surface deposition of serum components (26).

It is of interest further to inquire: (a) whether phagocytosis and agglutination occur at a constant critical surface potential difference or whether a factor or factors other than potential difference enter into the determination of phagocytosis and agglutination, and (b) whether phagocytosis and agglutination can be formulated in terms of the properties of the sensitizing substance alone or are dependent both on the properties of the sensitizing substance and on the intrinsic properties of the bacteria undergoing sensitization. These questions may be answered from the data in Table I.

Table I gives the cataphoretic velocities at which phagocytosis and agglutination began, at which phagocytosis reached 50 per cent and at which agglutination approached completeness. Phagocytosis and agglutination are seen to begin and to reach the levels indicated within

TABLE I.

The Relation of Cataphoretic Velocity to Agglutination and Phagocytosis.

1. Antigen	2. Time of immunization	3. Unsensitized antigen		4. Phagocytosis begins		5. Agglutination begins		6. Phagocytosis 50 per cent or more		7. Agglutination +++ or ++++		8. Minimum potential difference	
	weeks	*c.v. in μ/sec.*		*c.v. in μ/sec.*		*c.v. in μ/sec.*		*c.v. in μ/sec.*		*c.v. in μ/sec.*		*c.v. in μ/sec.*	
M. avium (Arloing strain)	1	1.92		1.18		1.73		1.50		1.18		1.18	
	2	1.67		1.50		1.50		1.34		1.30		0.92	
	3	1.96	1.83	1.82	1.55	1.82	1.49	1.55	1.34	0.89	0.88	0.49	0.69
	4	1.83	(23 mv.)	1.71	(20 mv.)	1.55	(19 mv.)	1.00	(17 mv.)	0.61	(11 mv.)	0.43	(9 mv.)
	6	1.67		1.45		1.00		1.32		0.57		0.49	
	9	1.93		1.64		1.32				0.75		0.63	
M. tuberculosis (Bovine III strain)	1	1.37		0.92		1.34		Not reached		Not reached		0.92	
	2	1.37	1.57	1.13	1.22	1.34	1.36	0.83	0.80	Not reached		0.83	0.72
	3	1.98	(20 mv.)	1.55	(15 mv.)	1.55	(17 mv.)	0.58	(10 mv.)	Not reached		0.58	(9 mv.)
	4	1.56		1.29		1.23		0.98		Not reached		0.54	
M. chelonei	1	1.54		1.43		1.45		Not reached		1.43		1.43	
	2	1.36		1.09		1.09		1.04		1.04		0.54	
	3	1.24	1.44	1.17	1.29	0.95	1.21	0.95	1.02	0.65	1.04	0.26	0.59
	4	1.29	(18 mv.)	1.15	(16 mv.)	1.15	(15 mv.)	0.89	(13 mv.)	0.89	(13 mv.)	0.41	(7 mv.)
	7	1.78		1.59		1.41		1.20		1.20		0.32	
M. avium (Prague strain)	1	1.01		0.83		0.83		Not reached		Not reached		0.56	
	2	1.05		0.75		0.82		0.60		0.60		0.60	
	3	1.18	1.09	1.04	0.89	1.17	0.99	0.71	0.69	0.66	0.64	0.66	0.63
	4	1.10	(14 mv.)	0.78	(11 mv.)	1.22	(12 mv.)	0.57	(9 mv.)	0.52	(8 mv.)	0.52	(8 mv.)
	7	1.13		1.07		0.93		0.89		0.80		0.80	

In columns 1 and 2 are given the antigen and the duration of immunization at the time the sensitizing serum was drawn; in column 3 the cataphoretic velocity (c.v.) of the control unsensitized antigen in salt solution is given.

In columns 4 and 5 are given the cataphoretic velocities corresponding to the beginning of phagocytosis and agglutination.

In columns 6 and 7 the cataphoretic velocities are given when phagocytosis has reached 50 per cent or over, and agglutination +++ to ++++.

The last column shows the lowest cataphoretic velocity reached by the antigen in each experiment.

The values in each category are averaged and the mean value is calculated as potential difference in millivolts.

The table shows that phagocytosis and agglutination occur within certain zones of cataphoretic velocity; but these zones are

fairly definite zones of surface potential difference,[3] but the potential zones are different for the different organisms. Consider for instance the Arloing and Prague strains of avian tubercle bacilli of similar growth habit; the Arloing strain has a relatively high surface potential difference (average, 23 millivolts), and the Prague a low potential difference (average, 14 millivolts). The phagocytosis and agglutination titers for the Arloing strain fall at 20 and 19 millivolts and for the Prague strain at 11 and 12 millivolts; phagocytosis of Arloing reached 50 per cent at about 17 millivolts, of Prague at about 9 millivolts; agglutination approached completeness with Arloing at about 11 millivolts, with Prague at about 8 millivolts. Yet in the highest concentrations of immune sera both strains were reduced to about the same potential difference.

We conclude, therefore, (a) that phagocytosis and agglutination do tend to occur within certain zones of surface potential difference; since, however, these zones are different for the different organisms it is obvious that another factor or factors than potential difference must enter into these phenomena.

Further consideration of Table I and of other data leads to the conclusion (b) that phagocytosis and agglutination cannot be formulated in terms of the properties of the sensitizing substance alone but depend both on the properties of the sensitizing substance or substances and on the intrinsic properties of the bacteria undergoing sensitization.

Evidence pointing to this conclusion may be summarized as follows: Agglutination of Bovine III never approached completeness although the surface potential difference of this strain was reduced well below the level required for +++ or ++++ agglutination of Arloing or the turtle bacillus (*chelonei*). This difficult agglutinability is characteristic for mammalian tubercle bacilli in general, and has given rise to a number of artifices, *e.g.*, agglutination at high temperature, agglutination of a substitute strain (Arloing strain), and the resuspension reaction (19). Phagocytosis of certain difficultly agglutinable strains of mammalian tubercle bacilli, on the other hand, may be high even without serum sensitization. This point is illustrated in Table

[3] Surface potential difference is measured by and is proportional to cataphoretic velocity.

TABLE II.

Atypical Phagocytosis of Human Tubercle Bacillus Sensitized with Aged Serum.

M. tuberculosis, strain H 37, in anti H 37 serum	Serum dilutions							
	1:4	$1:4^2$	$1:4^3$	$1:4^4$	$1:4^5$	$1:4^6$	$1:4^7$	Sodium chloride control
Agglutination	++	+ to tr.	+	tr.	+ to tr.	tr.	tr.	tr.
*Resuspension	+++	+++	+++	++ to +	+	++ to +	0	tr.
*Interface	More positive than control							
*Cataphoresis, μ/sec	0.70	1.16	1.87	2.12	2.14	2.14	2.28	2.31, 2.12
*Phagocytosis, *per cent*	5	17	88	81	85	89	88	71

* Bacteria sensitized and washed before testing.

II in which it is shown that a human tubercle bacillus of low virulence was phagocytized by 71 per cent of cells even without sensitization. Agglutination in the instance shown in Table II was slight although resuspension and cataphoresis showed the binding of agglutinin; the phagocytic prezone is characteristic of the aged serum used for sensitization; (see paper following). Tests with several other cultures of human tubercle bacilli gave 50 per cent or higher phagocytosis in the unsensitized 0.85 per cent sodium chloride suspension controls. Phagocytosis of this high order in the unsensitized controls has not been found with any type of acid-fast bacteria other than the human tubercle bacillus, and has not been found with all cultures of the human bacillus. It has already been recorded that the human tubercle bacilli we have studied in the interface differ from other types of acid-fast bacteria in their wetting properties (27).

Phagocytosis and agglutination are thus dependent both upon the properties of the sensitizing serum substance or substances and upon the intrinsic properties of the bacteria themselves.[4] Serum sensitization tends to promote both phagocytosis and agglutination. The intrinsic properties of the bacterium may be either favorable or unfavorable to phagocytosis or agglutination and may even influence the 2 phenomena in opposite directions. Since agglutination is a matter of the cohesion of the bacteria themselves, and phagocytosis necessitates adhesion of the bacteria to, and engulfing by, leucocytes, the relative independence of the influence of the original intrinsic bacterial properties on the 2 phenomena is not surprising.

SUMMARY.

Methods are described for investigating the relation between phagocytosis of bacteria by polymorphonuclear leucocytes, and certain physical-chemical properties of the bacterial surface.

Serum sensitization causes the following changes in the properties of acid-fast bacteria: (a) increased cohesiveness, (b) decrease in

[4] However it is possible that with maximum sensitization by a strong homologous serum the deposited sensitizing substance may form a complete film enveloping the sensitized bacterium. We are not sure whether the sensitizing film ever becomes complete or not. If it does, the original properties of the bacterial surface would doubtless no longer be factors in agglutination and phagocytosis.

surface electric potential difference, (c) decrease in wettability of the bacteria by oil, and (d) increased phagocytosis.

Tests have been conducted periodically with the sera of 4 rabbits under active immunization with as many strains of acid-fast bacteria; the parallelism between the alteration in bacterial surface properties and the promotion of phagocytosis by these sera has been, within the experimental error, complete.

The percentage of phagocytosis of a given bacterial suspension has been found to depend both upon the sensitizing serum component or components deposited upon the bacterium and upon the intrinsic properties of the unsensitized bacterial surface.

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ON THE MECHANISM OF OPSONIN AND BACTERIOTROPIN ACTION.

II. Correlation between Changes in Bacterial Surface Properties and in Phagocytosis Caused by Normal and Immune Sera.

By BALDUIN LUCKÉ, M.D., MORTON McCUTCHEON, M.D., MAX STRUMIA, M.D., and STUART MUDD, M.D.

(*From the Department of Pathology, and the Henry Phipps Institute, University of Pennsylvania, Philadelphia.*)

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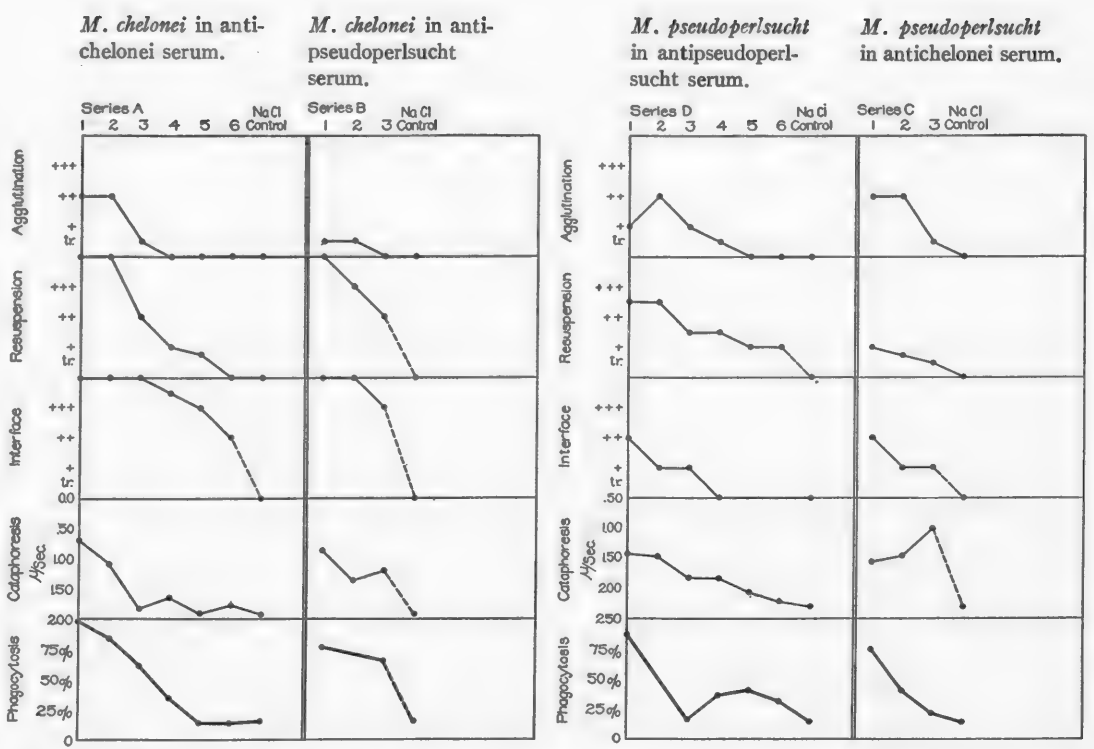
The sera of rabbits under immunization have been shown in the preceding paper (1) to cause parallel effects on the surface properties and the phagocytosis of acid-fast bacteria. Is this correspondence between the serum effects on surface properties and on phagocytosis a constant and necessary relation, or do circumstances exist in which the two effects are dissociated? It can be said at once that, in our experiments at least, whenever a serum has promoted phagocytosis it has invariably also altered the properties of the bacterial surfaces; and the surface and phagocytic effects have been in rough quantitative correspondence at least. Exceptionally circumstances have been found in which sera have altered bacterial surface properties, but have not increased phagocytosis. These failures of correspondence have all been with heated or aged sera or with sera of another species than the species from which the leukocytes were obtained.

Bacteriotropins.

Freshly drawn immune sera have regularly shown parallel bacteriotropic and bacterial-surface effects. An illustrative experiment is given in Text-fig. 1. The intensities of the several reactions are plotted as ordinates, and the final serum dilutions as abscissæ. It is seen that each serum when acting on its homologous strain caused increased cohesiveness (as indicated by the positive resuspension and interface



TEXT-FIG. 1. Homologous and cross reactions between turtle and pseudoperlsucht bacilli and their corresponding rabbit immune sera. Fresh sera heated to 56°C. for 30 minutes. Broken lines indicate that the titer was not reached. Numbers on horizontal axis indicate serum dilutions in powers of 4; thus 3 indicates a dilution of $1:4^3$ or 1:64. There is a general correspondence between bacterial surface changes and phagocytosis.

reactions), altered wetting properties (interface reaction), decreased surface potential difference (cataphoresis), and increased phagocytosis. The antipseudoperlsucht serum caused strong surface and bacteriotropic effects when used with the turtle bacillus (*M. chelonei*), and antichelonei serum gave moderate surface and bacteriotropic effects with the pseudoperlsucht bacillus.

The familiar fact that bacteriotropic action is exerted upon the bacterium not upon the leucocyte is demonstrated in Table I. In this experiment the bacteria were sensitized and then washed and suspended in saline solution. It is seen that phagocytosis is efficiently promoted by sensitization even in the absence of the sensitizing serum. The strains used were an avian and a bovine tubercle bacillus. Cross reactions are here almost as strong as those between homologous antigen and antibody; and again bacterial surface changes and increase in phagocytosis are in close correspondence.[1]

Aged Immune Sera.—Rabbit immune sera which had been kept in the ice-box without preservative for about 16 to 18 months were kindly put at our disposal by Dr. J. D. Aronson. These had been kept in large test tubes stoppered with cotton; accordingly they had undergone some evaporation. Moreover a heavy greyish precipitate had formed in the bottom of each, and most of the sera showed some opalescence which could not be cleared by centrifugation. Typical effects produced by such sera are shown in Text-figs. 2 and 3.

Prague bacillus treated with homologous antiserum exhibits characteristic surface changes (Series A), (Text-fig. 2). Phagocytosis is also increased but only in high dilutions of serum, reaching its maximum at approximately 1:1000 and 1:4000 dilution. This surprising phagocytic prezone cannot be explained satisfactorily by postulating injury of the leucocytes by the aged serum, as is shown by Series C, in which the same anti-Prague serum caused a moderate increase in phagocytosis without prezone, with milk bacillus, in correspondence with moderate bacterial surface changes.[2] The anti-milk-bacillus serum with homologous antigen caused strong bacterial surface

[1] A graph of this experiment has appeared in Colloid symposium monographs (2).

[2] The tubes of Series C were contaminated with a coccus. This may have been responsible for the prezone in the resuspension reaction, which is not a usual finding.

TABLE I.

Homologous and Cross Reactions between M. avium (Prague Strain) and M. tuberculosis (Bovine III Strain) and Their Corresponding Rabbit Immune Sera. Serum Dilutions Are Given as Powers of 4.

In this experiment sensitized bacteria were washed before phagocytosis. It is seen that even in the absence of free serum there was a high degree of phagocytosis.

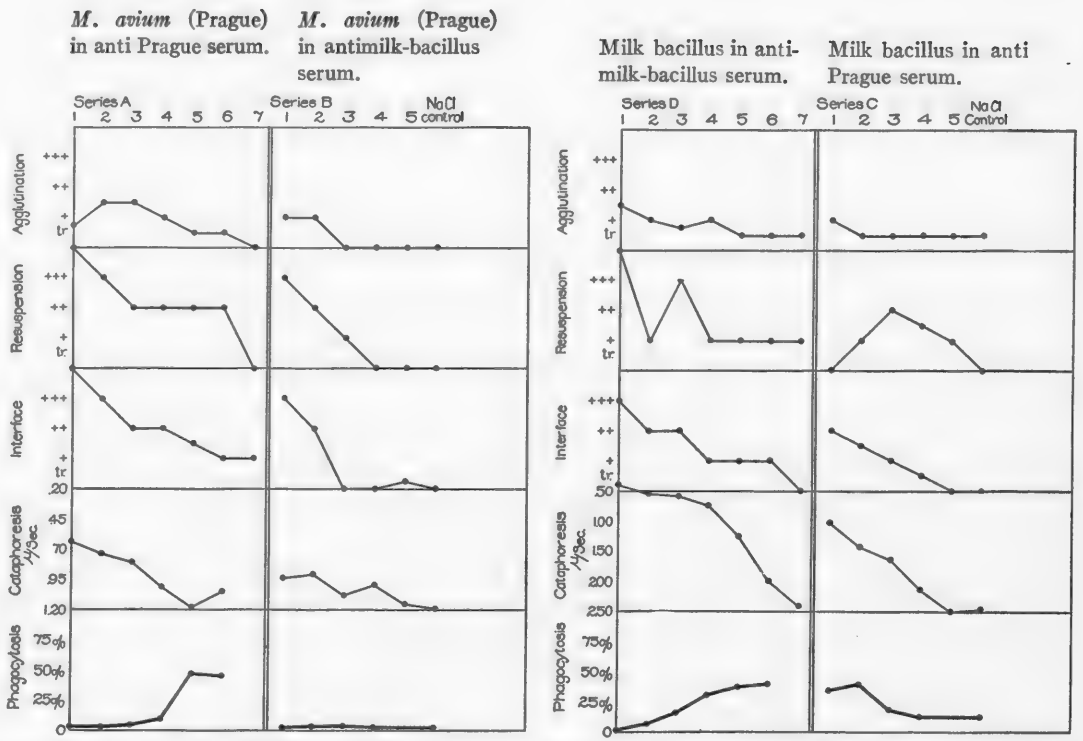
	Serum dilutions								Sodium chloride control
	1:4	1:16	1:64	1:256	1:1024	1:4096	1:16,384	1:65,536	
Series A. Prague in Anti-Prague Serum.									
Agglutination	++++ to +++	++++ to +++	++++ to +++	+	sl. tr.	sl. tr.	sl. tr.	sl. tr.	sl. tr.
Resuspension	++++	++++	+++	++	0	tr.	0	0	0
Interface	++++	++++	++ to +	+	tr.	0	—	—	0
Cataphoresis, μ/*sec.*	0.34	0.21	0.45	0.70	0.83	0.86	0.93	—	1.02
Phagocytosis, *per cent*	92	97	99	94	52	7	7	6	3
Series B. Prague in Anti-Bovine III Serum.									
Agglutination	++++ to +++	++++ to +++	+++	+	+ to tr.	tr.	—	—	sl. tr.
Resuspension	+++ to ++	++++ to +++	+++ to ++	++ to +	0	0	—	—	0
Interface	+++	+	tr.	0	—	—	—	—	0
Cataphoresis, μ/*sec.*	0.24	0.39	0.65	0.79	0.89	0.91	—	—	0.89, 0.98
Phagocytosis, *per cent*	73	64	79	72	44	20	—	—	4

Series C. Bovine III in Anti-Prague Serum.

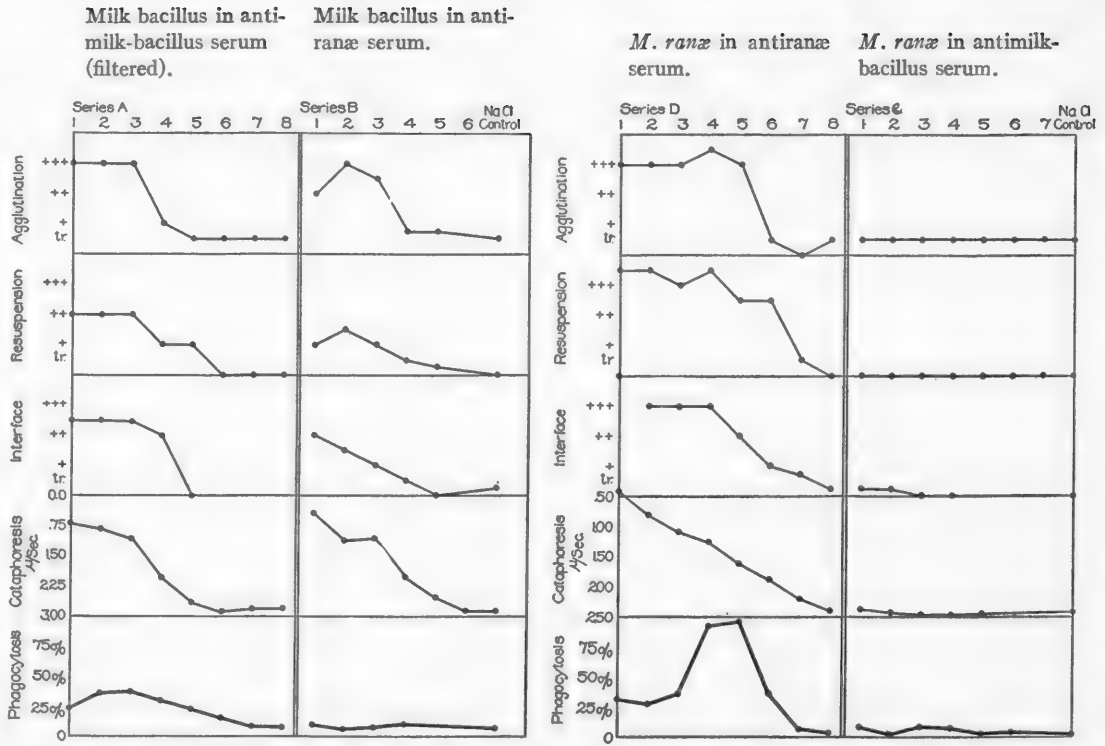
Agglutination	+++ to ++	+++ to ++	++	+	sl. tr.	sl. tr.	—	—	sl. tr.
Resuspension	++++	+++	++	+	0	0	—	—	0
Interface	+++	+++	++	+	tr.	0	—	—	0
Cataphoresis, μ/*sec.*	0.35	0.47	0.92	1.40	1.33	1.53	—	—	1.52
Phagocytosis, *per cent.*	44	46	28	43	35	9	—	—	3

Series D. Bovine III in Anti-Bovine III Serum.

Agglutination	++++ to +++	++++ to +++	+++	++	sl. tr.	sl. tr.	sl. tr.	sl. tr.	sl. tr.
Resuspension	++++	++++	+++	++	0	0	0	0	0
Interface	++++	+++	+++ to ++	++ to +	+	tr.	0	—	0
Cataphoresis, μ/*sec.*	0.08	0.17	0.31	1.18	1.17	1.53	1.46	1.50	1.69
Phagocytosis, *per cent.*	47	44	45	20	31	15	7	4	6



TEXT-FIG. 2. Homologous and cross reactions between an avian tubercle bacillus and a saprophyte and their corresponding rabbit immune sera. Sera aged about 16 months. Symbols as in Fig. 1. Note phagocytic prezones.



TEXT-FIG. 3. Homologous and cross reactions between frog and milk bacilli and their corresponding rabbit immune sera. Sera aged about 16 months, and filtered before use. Symbols as in Fig. 1. Note phagocytic prezones.

changes and enhanced phagocytosis, but again with a striking phagocytic prezone. This serum, on the other hand, failed to increase phagocytosis of Prague bacillus, although it caused moderate surface changes (Series B).

Anti-milk bacillus serum from the same source as in Text-fig. 2 was passed through a Seitz asbestos laboratory filter disc and used in the experiment represented by Text-fig. 3. It is seen that the percentage phagocytosis caused by the filtered serum is somewhat less than that caused by the unfiltered, and the phagocytic prezone has been almost abolished by the filtration (Series A). The milk bacillus serum was practically without effect either on the surface properties or the phagocytosis of frog bacillus (*M. ranæ*, Series C). Anti-frog-bacillus serum, which also had been filtered through a Seitz disc, caused surface changes and increased phagocytosis, with prezone, with its homologous antigen (Series D). The anti-frog bacillus serum caused moderate surface reactions but no increased phagocytosis with the heterologous organism (Series B).

Two conditions have been found, then, in which correspondence may fail between the effects produced by aged sera on the bacterial surface properties and on phagocytosis. Save for them the correspondence of surface and phagocytic effects has been satisfactory even with aged immune sera. These conditions are:

1. The aged sera in all of 13 homologous series gave phagocytic prezones. Maximum phagocytosis was reached in serum dilutions which might be as high as 1:1000; phagocytosis in the lesser serum dilutions was far below the maximum or even absent. Prezones comparable to this were not found with the surface reactions.

2. The aged sera sometimes produced surface changes but no phagocytosis when reacting with heterologous bacterial strains. Four out of 11 series gave this result; in the other 7 heterologous series correspondence between bacterial surface changes and phagocytosis was satisfactory.

The Phagocytic Prezone with Aged Immune Sera.—Phagocytic prezones occurred, as has been said, in all of the homologous series with the aged immune sera; only one prezone occurred in a heterologous series. Moreover we have two clear experiments in which an aged serum gave a striking prezone with its homologous bacterial strain,

but caused phagocytosis without prezone with a heterologous strain. The first example is given by Series A and C of Text-fig. 2. The second is given by Series A and C of Table II. Series B and D of Table II seem to be a third example, but this is less clear because of the irregularity of the phagocytosis in Series D.

The results just cited indicate that the phagocytic prezone cannot be chiefly due to injury of the leucocytes by the aged sera or to other non-specific effect, but must be an expression of the properties of the specific combination of bacteria and sensitizing substances. The evidence leading to this conclusion is strengthened by the observation that a phagocytic prezone may persist after washing the sensitized bacteria. In 4 series in which the sensitized bacteria were washed, some prezone persisted in 3; in the fourth series phagocytosis was practically abolished by washing. Two of the persistent prezones were, however, considerably diminished by the washing; one of these is shown in Table III.

It is important to bear in mind that these phagocytic prezones with aged sera, however interesting and perplexing from a theoretical standpoint, are a distinctly artificial phenomenon. Such prezones have practically not occurred, at least in our experience, with sera used within a few days of drawing the blood, whether the sera have been heated to 56°C. for 30 minutes or have been used unheated. Such fresh sera have almost invariably shown phagocytosis at or near maximum in the highest serum concentration used in this work, namely a final concentration of 25 per cent serum.

The most marked prezone found with freshly prepared sera occurred in Rabbit 20-02, at the end of its 3rd week of immunization with Arloing bacillus (1); the percentages of phagocytosis in the successive tubes in this experiment (the dilution factor was 0.25 as usual) were 67, 72, 89, 97, 81, 26, 10, 11, and control 11 per cent. This prezone was not present with the serum of the same rabbit the week before or after this experiment. Moreover the leucocytes were noted as agglutinated in the first tubes of this series; this probably explains the apparent prezone.

There are good grounds for supposing that the protective power of some immune sera depends largely upon their efficiency in promoting phagocytosis (3). The abnormal bacteriotropic behavior of the aged sera used in our experiments suggests a danger in dispensing old antibacterial sera for clinical use.

TABLE II.

Homologous and Cross Reactions between M. avium (Arloing Strain) and M. tuberculosis (Bovine III Strain) and Their Corresponding Rabbit Immune Sera.

Prezones in phagocytosis are shown in homologous reactions but not in cross reactions.

	Serum dilutions							Sodium chloride control
	1:4	1:16	1:64	1:256	1:1024	1:4096	1:16,384	
Series A. Arloing in Anti-Arloing Serum.								
Agglutination	+++	+	+++	+++	+	0		0
Resuspension	+++ to ++	+++	++++	+++ to ++	+ to tr.	tr.		0
Interface	++	++++	+++ to ++++	++	+	tr.		0
Cataphoresis, μ/*sec.*	1.27	1.73	1.80	1.96	1.65	1.91		1.64
Phagocytosis, *per cent.*	22	22	72	96	81	40	11	8
Series B. Arloing in Anti-Bovine III Serum.								
Agglutination	+++	+++	+++	++	0	0		0
Resuspension	++++ to +++	++++ to +++	+++	tr.	0	0		0
Interface	+++	++	++ to +	+	+ to tr.	0	0	0
Cataphoresis, μ/*sec.*	0.95	1.20	1.47	1.81	1.80			1.77
Phagocytosis, *per cent.*	53	26	25	9	11	10		—

Series C. Bovine III in Anti-Arloing Serum.

Agglutination.....	+	+	tr.	0	0	0		0
Resuspension.....	++	+	++	+ to tr.	0	0		0
Interface.....	+++	++ to +++	++	++	tr.	0		0
Cataphoresis, μ/*sec*.....	1.54	1.16	1.15	1.52	1.26			1.54
Phagocytosis, *per cent*..	24	19	10	12	13	9		1

Series D. Bovine III in Anti-Bovine III Serum.

Agglutination.....	+	+	+	+	tr.	0		0
Resuspension.....	+	++	++	++	++ to +	tr.		0
Interface.....	++	+++	+++	+++	+	tr.		0
Cataphoresis, μ/*sec*....	0.97	0.94	1.11	1.69	1.14	1.25	1.33	1.64
Phagocytosis, *per cent*..	15	11	14	11.5	29	3	2	—

TABLE III.

The Effect on Surface Properties and on Phagocytosis of Washing Bacteria Sensitized with Aged Serum. M. avium (Arloing Strain) Was Sensitized with Homologous Aged Rabbit Serum. In Series A Determinations Were Made in the Presence of Serum Dilutions; in Series B Sensitized Bacteria Were Washed to Remove Free Serum.

It is seen that the intensity of the phagocytic prezone is diminished by washing the bacteria.

	Serum dilutions							Sodium chloride control
	1:4	1:16	1:64	1:256	1:1024	1:4096	1:16,384	
Series A (in Serum Dilutions)								
Agglutination	+++ to ++	+++ to ++	+++	++++ to +++	tr. to 0	0	0	0
Resuspension	++	++	+++	++++	+	tr.	0	0
Interface	+++	+++	++++	++	+	+		0
Cataphoresis, μ/*sec.*	0.93	1.47	1.69	1.76	1.50	1.42	1.45	1.61
Phagocytosis, *per cent*	25	28	69	90	78	45	7	8
Series B (Sensitized, Washed)								
Agglutination	—	—	—	—	—	—	—	—
Resuspension	++ to +	++	+++	+++	++ to +	+ to 0	0	0
Interface	+++ to ++	++++	++++ to +++	+++	+ to tr.	±	0	±
Cataphoresis, μ/*sec.*	0.28	0.33	0.55	1.25	1.42	1.57	1.63	1.64
Phagocytosis, *per cent*	55	54	62	65	85	73	32	—
Series C (Sensitized in 1:256 Serum, Then Made Up in Serum Dilutions)								
Phagocytosis, *per cent*	30	29	68	87	93	93	85	—

Opsonins.

Rabbit Sera.—With fresh, unheated sera of normal rabbits there has been excellent parallelism between the bacterial surface changes and opsonization. Bacterial cohesion has been increased, surface potential difference reduced, wetting properties altered and the bacteria have been prepared for phagocytosis. The serum concentrations required to give these effects are of course much higher than with immune sera.

The lowest concentrations of normal serum which promoted phagocytosis were for sensitized, washed bacteria: 1:5, 1:5, 1:5, 4:5, 1:5, 4:5, and 1:5; one series in which the bacteria were washed failed to give phagocytosis even after sensitization with 80 per cent serum. For bacteria exposed to leucocytes in the presence of fresh normal sera the titers were somewhat higher, *i.e.*, 1:16, 1:16, 1:64 and 1:16. The resuspension and cataphoresis titers have run on the average a little higher than the phagocytosis titers, the interface titers about the same, although all titers with these concentrated normal sera have tended to be irregular.

With the sera of normal rabbits inactivated by heating to 56°C. for 30 minutes correspondence between bacterial surface changes and phagocytosis has not been good. In 7 series of bacteria sensitized with heated normal sera and washed, phagocytosis was not induced in any, whereas the surface changes were present, although to a less degree than after sensitization with unheated serum. In 28 series in which the bacteria were exposed to leucocytes in the presence of heated normal rabbit serum phagocytosis was induced in only about 16 series, whereas the bacterial surface properties were altered to some degree in practically all.

Human Sera.

Bacteria have been sensitized with fresh human sera, washed with 0.85 per cent sodium chloride solution, and tested for surface changes and for phagocytosis by rabbit leucocytes. The sera tested were from 7 non-tuberculous individuals, from 5 cases of pulmonary and 6 of bone or joint tuberculosis, as indicated in the protocols below. The characteristic changes in bacterial surface properties were regularly produced by these sera. Qualitatively the changes were not distinguishable by the surface reactions from those produced by rabbit sera. Quantitatively the bacterial surface changes varied with the different

sera, but were usually a little greater than those produced by normal unheated rabbit sera. Yet in no case was a clearly demonstrable increase in phagocytosis by rabbit leucocytes caused by sensitization with human serum.

Patients with Pulmonary Tuberculosis.—(Sera made available through kindness of Dr. I. Kaufman.)

A. L., female. Pulmonary tuberculosis, moderately advanced, moderate symptoms, sputum positive; tuberculosis complement fixation, ++++; Wassermann negative. Unheated and heated serum tested in dilutions of 1:4, 1:16 and 1:64 with *M. tuberculosis* (Bovine III).

E. G., female. Pulmonary tuberculosis, moderately advanced, slight symptoms, sputum positive; tuberculosis complement fixation, negative; Wassermann negative; tuberculin, 0.00001 cc. O. T., ++. Unheated serum tested in dilutions of 1:4, 1:16, 1:64 and 1:256 with Bovine III.

M. C., female. Pulmonary tuberculosis with apical râles; far advanced; severe symptoms; sputum positive. Unheated and heated serum tested in dilutions of 1:4, 1:16 and 1:64 with Bovine III.

B. R., female. Pulmonary tuberculosis with apical râles; far advanced, moderate symptoms. Tuberculosis complement fixation, negative; Wassermann negative. Unheated and heated serum tested in dilutions of 1:4, 1:16 and 1:64 with Bovine III.

G. H., female. Apical tuberculosis; tuberculosis complement fixation ++++; tuberculin, 0.00001 cc. O.T., +. Wassermann ++++. Unheated serum tested in dilutions of 1:4, 1:16 and 1:64 with Bovine III.

Surgical Tuberculosis.—(Sera made available through kindness of Dr. George Wagoner.)

Sera of A. A. and C. P. tested unheated in dilutions of 1:4, 1:16, 1:64, 1:256 and 1:1024. Other sera tested, unheated, in dilutions of 1:4 and 1:16. All sera tested, heated, in dilutions of 1:4. Antigen for all series, *M. tuberculosis* (Bovine III).

A. A., male. Pott's disease of 4th, 5th, 6th and 7th dorsal vertebræ.

C. P., male. Pott's disease of 5th and 6th dorsal vertebræ.

I. L., male. Lumbo-sacral spondylitis, probably tuberculous, numerous sinuses, pulmonary tuberculosis (?).

J. S. Tuberculous left hip with sinuses; old pulmonary tuberculosis.

A. S. Tuberculous spine and shoulder with sinuses.

H. S. Tuberculous right wrist (?).

Non-Tuberculous Individuals.—

Sera from 6 persons who came into the chest clinic but were shown by physical and laboratory examination to be free from clinical tuberculosis. Serum from a healthy university student. Tests as above with Bovine III or an avian tubercle bacillus as antigen.

These experiments failed to give evidence that any of the reactions used afforded a satisfactory means of distinguishing the sera of tuberculous from those of non-tuberculous individuals.

It is to be emphasized that the bacteria sensitized with human sera were in all cases washed before testing for phagocytosis in order to exclude the possibility of a direct toxic effect of human serum on the rabbit leucocytes. The leucocytes were shown to be capable of phagocytosis by other series in the same experiments in which sensitization was produced by rabbit sera. A further experiment was performed in which bacteria were first sensitized with unheated normal rabbit serum, then washed and resuspended in 1:500 human serum before testing for phagocytosis. This concentration of human serum was considerably higher than the amount which could have remained after washing of the bacteria sensitized even with the highest concentrations of human serum. Phagocytosis of the bacteria sensitized with rabbit serum occurred nevertheless in the presence of the 1:500 human serum.

The rabbit polymorphonuclear leucocytes can then differentiate with precision bacteria sensitized with human serum from those sensitized with rabbit serum, whereas our physical-chemical tests fail to make this differentiation. We believe phagocytosis to be dependent upon the spreading of the leucocyte about the bacterium primarily under the action of surface forces (1). This capacity of a leucocyte to differentiate the sensitizing substances of two mammalian species[3] implies a very striking selectivity in the spreading or wetting requirements of the leucocyte, which, however, is not wholly without analogy. Thus sponges have been separated into their component cells by straining through cloth and the separated cells of different species have been mixed together. The cells creep about by amœboid motion until they meet and fuse with other cells of the same species. Syncytia composed of cells of each species but not of mixed species are

[3] However, we have obtained abundant phagocytosis by rabbit leucocytes of tubercle bacilli sensitized with hyperimmune antituberculous goat sera and washed with saline. Robertson and his collaborators obtained phagocytosis of pneumococci by rabbit leucocytes suspended in rabbit serum to which human serum containing antipneumococcus antibodies had been added (3). Normal serum added to such rabbit serum leucocyte mixtures did not cause phagocytosis (3, 4).

thus formed (5). The spreading of fluids, in general, is known to be to some extent selective, and to depend both on the properties of the spreading surface and the surface spread upon (6).

In the experiments with normal rabbit sera, then, excellent correspondence has been obtained between changes in bacterial surface properties and opsonic effects so long as fresh, unheated sera have been used. With heated normal rabbit serum or with human serum, heated or unheated, surface changes have been produced but phagocytosis has often failed. It would seem then that the requirements for spreading of the leucocyte surface are so delicate that discrimination occurs between sensitizing substances deposited on the one hand by fresh immune rabbit sera and fresh unheated normal rabbit sera and on the other hand by aged immune sera, heated normal rabbit sera and human sera. The fact may once again be emphasized that the failures of correspondence between bacterial surface changes and phagocytosis have all been failures of phagocytosis, and have all been found under extremely artificial conditions. Correspondence between changes in bacterial surface properties and increase in phagocytosis has been excellent when conditions *in vitro* have been made to imitate as nearly as possible conditions *in vivo*.

CONCLUSIONS.

The work reported in this and in previous papers (1, 7) demonstrates the following relations for acid-fast bacteria and rabbit polymorphonuclear leucocytes:

1. The combination of a substance or substances present in fresh immune rabbit serum, heated or unheated, or in fresh unheated normal rabbit serum, with a substance or substances in the bacterial surface causes an increase in cohesiveness, decrease in surface potential difference and characteristic alteration in wetting properties of the bacteria, and *prepares the bacteria for phagocytosis*.

2. (*a*) The effective substance or substances in the serum may become so altered as the result of heating or aging that combination with the bacterial surface, while causing changes in bacterial surface properties indistinguishable by the present physical-chemical tests from these just mentioned, *may not lead to phagocytosis*, or may lead to phagocytosis with a prezone not paralleled by a prezone in the changes in surface properties.

(*b*) Sensitization of bacteria with human sera causes changes in surface properties similar to those caused by rabbit sera, but does not lead to phagocytosis by rabbit leucocytes. The spreading requirements of rabbit polymorphonuclear leucocytes are evidently highly selective.

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ON THE MECHANISM OF OPSONIN AND BACTERIOTROPIN ACTION.

III. The Development and Effect of the Antibodies Found in Experimental Tuberculosis of Rabbits.

By MORTON McCUTCHEON, M.D., MAX STRUMIA, M.D., STUART MUDD, M.D., EMILY B. H. MUDD, and BALDUIN LUCKÉ, M.D.

(*From the Department of Pathology and the Henry Phipps Institute, University of Pennsylvania, Philadelphia.*)

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This study is concerned with three questions: first, the development of circulating antibodies in primary tuberculosis in rabbits; second, the development of antibodies in rabbits reinfected with tubercle bacillus while still incompletely recovered from a primary tuberculosis; and third, as in the two foregoing studies, with the correlation between the effects on bacterial surface properties and on phagocytosis produced by the sera of the animals studied.

The animals followed were taken from among those used by M. B. Lurie in continuation of his study of the fate of tubercle bacilli in the organs of the rabbit (1). Bacteriological data on these rabbits will be published later by Lurie. The titers of the sera of three groups of rabbits have been followed, each containing primarily infected as well as reinfected individuals. The sera were tested in all cases with suspensions in 0.85 per cent sodium chloride solution of a living bovine tubercle bacillus, Bovine III. This is an avirulent and now slightly atypical strain[1] (2). The test suspensions were adjusted to match a turbidity standard. The methods used are those described in a foregoing paper (3), except that the interface reaction was omitted. The sera were heated for 30 minutes at 56°C. in all cases.

[1] It was not practicable to use the infecting strain (Bovine C) as routine antigen in these tests because of the danger of infecting the workers. In a special experiment, however, living Bovine C bacilli were shown to exhibit typical surface changes and increase in phagocytosis as a result of serum sensitization. For all practical purposes Bovine III was an homologous antigen.

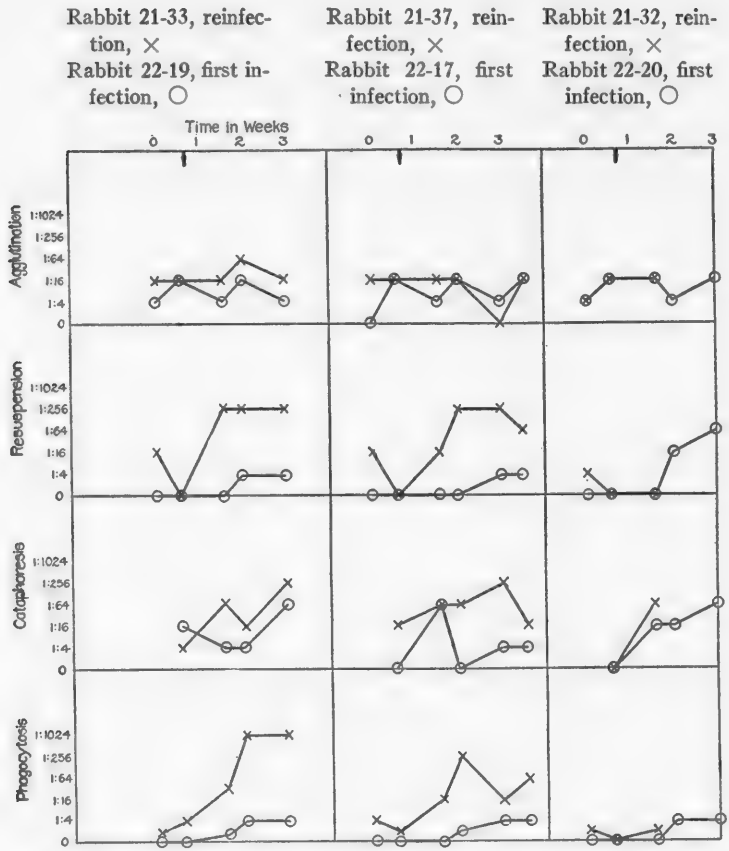

FIG. 1. Response in antibody production following a single injection (indicated by arrow at top of chart) of living, virulent tubercle bacilli. Three rabbits with residual foci of tuberculosis (crosses), and 3 previously healthy rabbits (circles). Ordinates represent the greatest dilutions of serum giving clearly positive reactions. It is shown that the reinfected animals promptly developed circulating antibodies strikingly in excess of those found in the course of primary infection. The changes in titer during tuberculous infection as indicated by the bacterial surface reactions (resuspension and cataphoresis) and by phagocytosis were in approximate correspondence.

Group 1.—Rabbits 21-32, 21-33 and 21-37 injected intravenously on May 17, 1928, with 0.001 mg. per kilo body weight of a living virulent human tubercle bacillus, Strain P-48A. X-ray of rabbits' lungs, Oct. 24, 1928, showed for Rabbit 21-32 slight, and for Rabbit 21-37 moderate disseminated pulmonary tuberculosis; lesions in Rabbit 21-33 were not certainly detectable by x-ray. These previously infected rabbits and normal Rabbits 22-17, 22-19, and 22-20 were injected intravenously on Nov. 6 with 0.01 mg. of a living virulent bovine tubercle bacillus, Bovine C. The titers of the sera of the rabbits of this group are given in Text-fig. 1; the injections on Nov. 6 are indicated by arrows at the top of the chart. The titers represent the greatest dilutions of serum giving clearly positive reactions; doubtful reactions and traces are omitted as in the first paper (3). Cataphoresis after sensitization with these relatively concentrated sera does not give a sharp end point; therefore in each experiment any value representing a change (decrease) of 20 per cent or more from the average cataphoretic velocity of the unsensitized bacteria of that experiment has arbitrarily been taken as positive.

The most striking feature of Text-fig. 1, and also of the charts following, is the difference in response to injection of the reinfection and first infection animals. The titers of the two groups do not differ greatly before the injections of Nov. 6, although those of the infected animals tend to be a little higher. But a marked difference appears at the first bleeding 6 days after injection; the titers of reinfection Rabbits 21-33 and 21-37 have clearly increased, whereas those of the first infection rabbits are essentially unchanged. Reinfection Rabbit 21-32, whose titers showed little change at the first bleeding on Nov. 12, was in bad condition at the time and died Nov. 13.

An increase in titers appeared in each of the first infection animals at the second (Nov. 15) or third (Nov. 22) bleeding, respectively 9 and 16 days after the injections. Although slight, this increase was so uniform in the 3 animals that we believe it to have been significant.

The potency of the sera in changing the properties of the bacterial surfaces (resuspension and cataphoresis reactions) and in promoting phagocytosis are parallel, within the experimental error, as was found to be the case with sera of animals under active immunization (3). The "agglutination" reaction, shown in the uppermost lines, does not render a satisfactory account of the changes in the sera shown by the other reactions. It has already been pointed out that the ordinary agglutination technique is not satisfactory with mammalian tubercle bacilli as antigen (4, 5), but that the resuspension reaction is a satisfactory detector of the binding of agglutinins by these bacilli. Our

experiments thus afford strong corroborative evidence of the parallelism of agglutinin and bacteriotropin content in sera (6, 7), not evidence contradicting such parallelism as might appear on first inspection of the charts.

The sera of Rabbits 21-33 and 21-37, 16 days after reinfection, *i.e.*, when active against Bovine III, were tested for specificity against *M. chelonei* (turtle bacillus). They did not react to a significant degree with *M. chelonei*.

The autopsy protocols of the rabbits of this group are given below.

Autopsies by M. B. Lurie.

Rabbit 21-32, died Nov. 13, 1928, *i.e.*, 7 days after *reinfection*. Lungs: moderate number of discrete, rather firm subpleural tubercles. Liver, spleen and bone-marrow: no tubercles. Kidney: discrete tubercles in cortico-medullary region.

Rabbit 21-37, killed Dec. 18, 1928, *i.e.*, 42 days after *reinfection*. Lungs: posterior portions have large caseous pus-containing cavities; anterior portions show discrete subpleural tubercles. Liver and bone-marrow: no tubercles. Spleen: enlarged, surface irregular, no clear-cut tubercles. Kidney: moderate number of cortical tubercles.

Rabbit 21-33, killed Jan. 8, 1929, *i.e.*, 63 days after *reinfection*. Lungs: a few scattered fibrocaseous tubercles, 2 to 3 mm. in diameter. Spleen: enlarged and mottled, no tubercles. Kidney, liver and bone-marrow: no tubercles.

Rabbit 22-20, killed Dec. 3, 1928, *i.e.*, 27 days after *first infection*. Lungs: numerous miliary tubercles about 2 mm. in diameter with punctate caseous foci. Liver: minute punctate tuberculous foci. Spleen: numerous subcapsular tubercles about 1 mm. in diameter. Kidney: moderate number of cortical tubercles about 1 mm. in diameter. Bone marrow: a few discrete tubercles.

Rabbit 22-17, killed Jan. 10, 1929, *i.e.*, 65 days after *first infection*. Lungs: anterior portions almost entirely consolidated (diffuse caseous pneumonia); posterior portions show massive conglomerate miliary tuberculosis; extensive communicating caseous foci. Liver: appears normal. Spleen: several tubercles 3 to 4 mm. in diameter. Kidney: moderate number of discrete cortical tubercles. Bone-marrow: no tuberculosis.

Rabbit 22-19, killed Jan. 11, 1929, *i.e.*, 66 days after *first infection*. Lungs: massive conglomerate miliary tubercles, 1 to 10 mm. in diameter. Spleen: about 4 times normal in size; numerous reddish subcapsular tubercles, 2 to 3 mm. in diameter. Liver: a few minute tubercles. Kidney: moderate number of cortical and corticomedullary tubercles. Bone-marrow: ill-defined tubercles.

Two of the reinfection rabbits, Nos. 21-32 and 21-37, showed a moderate number of discrete lesions in lungs and kidney, in all probability residual from the primary infection with tubercle bacilli of

human type. In the third reinfection rabbit, No. 21-33, the primary infection had been almost overcome, leaving only a few scattered fibro-caseous tubercles in the lung. All 3 animals were highly resistant

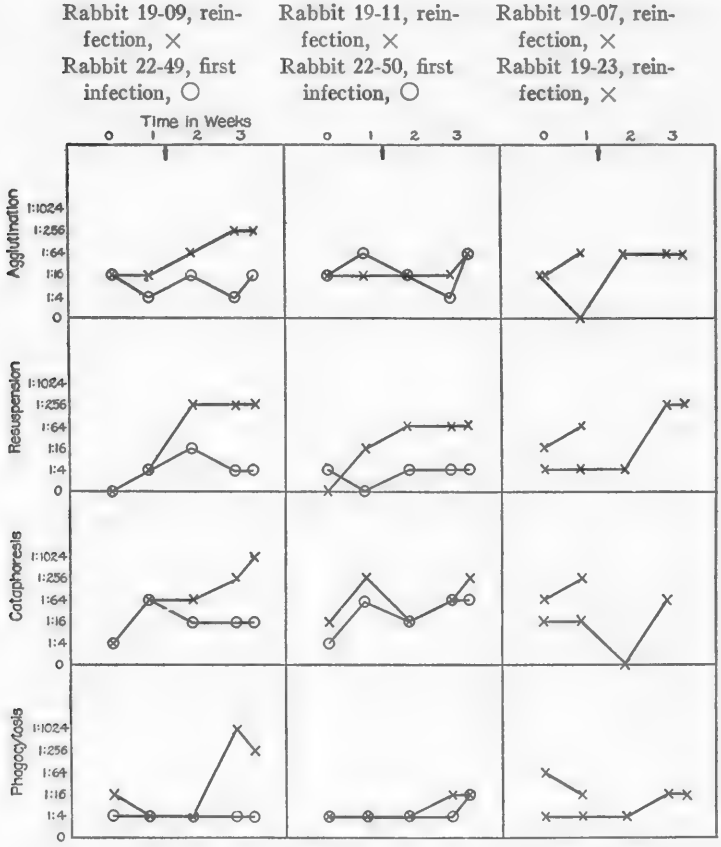

FIG. 2. Antibody production following single injection (indicated by arrow at top of chart) of living, virulent tubercle bacilli. Four rabbits with residual foci of tuberculosis (crosses), and 2 previously healthy rabbits (circles).

to the secondary infection with bovine tubercle bacilli; no lesions certainly referable to the second infection were found.

A marked contrast to the reinfection animals was afforded by the

rabbits infected with bovine tubercle bacilli without previous infection with bacilli of human type. In these the bovine infection ran its usual malignant course, causing after 2 months massive confluent tuberculosis of the lungs and involvement of the abdominal organs.

Group 2.—Rabbits 19-07, 19-09, 19-11 and 19-23 injected intravenously on Feb. 3, 1928, with 0.001 mg. per kilo body weight of a living, virulent, human tubercle bacillus, Strain P-48 A. These infected rabbits and normal Rabbits 22-49 and 22-50 injected intravenously on Oct. 11, 1928, with 0.01 mg. of living virulent bovine tubercle bacillus, Bovine C. Injections on Oct. 11 indicated in Text-fig. 2 by arrows at top of chart. In Groups 2 and 3 cataphoresis values are taken as positive if they represent a decrease of 10 per cent or more from the average cataphoretic velocity of the unsensitized bacteria of the experiment in question.

The increase in rate and quantity of antibody production in the reinfection rabbits as compared with the first infection rabbits again appears in Text-fig. 2. This difference is marked between Rabbits 19-09 and 22-49, less apparent between Rabbits 19-11 and 22-50. The reinfection rabbits in each case showed increase in titer by 11 days after injection; this increase was not consistently apparent 4 days after injection. No unequivocal increase in the titers of the 2 first-infection rabbits was detected within the 2 weeks after injection during which the experiment was continued.

Rabbit 19-07 died 3 days after reinfection. The titers before reinfection were unusually high. Also the amount of pulmonary disease was unusually great for rabbits infected with so small a dose of human bacilli. This association may have been coincidental, however.

The serum of Rabbit 19-11, 18 days after reinfection was tested for specificity against *M. chelonei*. It did not react significantly with *M. chelonei*.

Autopsies by M. B. Lurie.

Rabbit 19-07, died Oct. 14, 1928, *i.e.*, 3 days after *reinfection*. Lungs: extensive tuberculosis with cavitation; confluent subpleural lesions toward anterior margin. Pneumonia superadded in left lung. Liver: no tubercles. Spleen: no tubercles. Kidneys: 2 isolated tubercles in each.

Rabbit 19-09, killed Oct. 26, 1928, *i.e.*, 15 days after *reinfection*. Lungs: fibrocaseous consolidation of anterior margins. Lung elsewhere has a few discrete lesions. Liver, no tubercles. Spleen: enlarged and studded with firm grayish nodules. Kidney, no tubercles. Bone marrow: epiphysis of right tibia, caseous focus.

Rabbit 19-11, killed Nov. 8, 1928, *i.e.*, 28 days after *reinfection*. Lungs: moderate number of small glossy tubercles, 1 mm. in diameter, in both lungs; no caseation. Liver: fibrosis, but no tubercles. Spleen: enlarged; no gross tubercles. Kidneys: small number of pin-point tubercles. Bone marrow: no tubercles.

Rabbit 19-23, killed Nov. 23, 1928, *i.e.*, 43 days after *reinfection*. Lungs: numerous discrete, subpleural tubercles, 3 to 4 mm. in diameter with ill defined caseous centers. Liver: no tubercles. Spleen: no tubercles. Kidneys: small number of ill-defined cortical tubercles. Bone marrow: no tubercles. Fat depots remarkably well filled.

Rabbit 22-50, killed Nov. 21, 1928, *i.e.*, 41 days after *first infection*. Lungs: massive conglomerate miliary tubercles, 3 to 4 mm. in diameter, with marked caseation in centers. Liver: punctate tubercles. Spleen: extensive miliary tuberculosis. Kidney: moderate number of cortical tubercles with central punctate caseation. Bone marrow: moderate number of tubercles.

Rabbit 22-49, killed Dec. 12, 1928, *i.e.*, 62 days after *first infection*. Lungs: massive, conglomerate, miliary tubercles with conspicuous caseous centers; almost complete solidification. Liver: 1 or 2 questionable pin-point foci. Spleen: riddled with tubercles about 2 mm. in diameter. Kidneys: extensive cortical tubercles, 3 to 4 mm. in diameter, raised as hemispheres above the surface; tubercles have conspicuous caseous centers. Bone marrow: several discrete tubercles about 3 mm. in diameter. Animal well preserved.

Group 3.—Rabbits 16-13, 16-14, 16-20 and 16-22 injected intravenously on Oct. 17, 1927, with 0.001 mg. per kilo body weight of a living virulent human tubercle bacillus, Strain P-48A. Rabbits 16-13 and 16-14 and normal Rabbits 20-93 and 20-94 were injected intravenously on Apr. 18, 1928, with 0.01 mg. of living P-48A. Rabbit 16-22 and normal Rabbits 20-95 and 20-97 were injected intravenously on Apr. 18, and Rabbit 16-20 on Apr. 20, 1928, with 0.01 mg. of a living virulent bovine tubercle bacillus, Bovine C. Injections on Apr. 18 and 20 indicated in Text-figs. 3 and 4 by arrows at top of chart.

The rabbits of Group 3, (Text-figs. 3 and 4) were followed for a longer time, 6 or 7 weeks, after injection. Unfortunately the titrations made before injections are not available for comparison and are not indicated in the figures; human tubercle bacilli (P-48A), heated to 56°C. for 75 minutes, were used as antigen in the first titrations, and proved not to be satisfactory detectors. In the titrations plotted living Bovine III was used as antigen as in the other groups.

The increased antibody content of the sera of the reinfection rabbits as compared with the first infection rabbits is strikingly manifest in the majority of the titrations 7 days after reinfection and persists throughout the experiment. Although the titrations were quite irregular in this group, the difference between reinfection and first infection animals was brought out both by the bacterial surface re-

actions and by phagocytosis. Surface changes and phagocytosis again seem to be essentially concordant within the rather large experimental error.

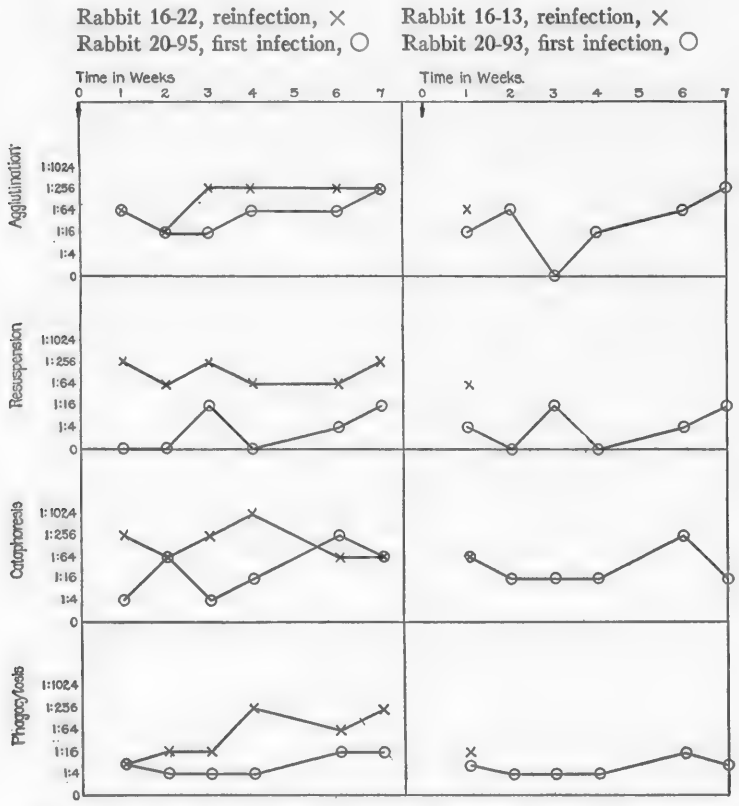

FIG. 3. Antibody production following a single injection (indicated by arrow at top of chart) of living, virulent tubercle bacilli. Two rabbits with residual foci of tuberculosis (crosses), and 2 previously healthy rabbits (circles).

The titers of the first infection animals seem to show a slight but nevertheless definite upward trend especially during the latter weeks of the experiment. Supporting data for this conclusion are afforded

by a comparison of the titrations of the sera of these first infection rabbits on the 6th and 7th week after infection with the titrations of 6 normal rabbits included in the same experiments. The results of

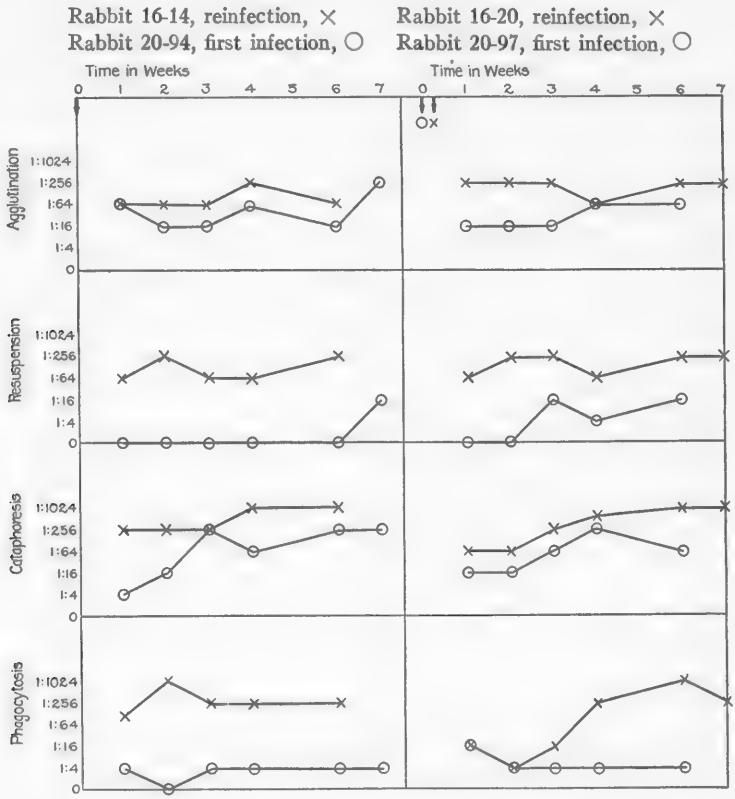

FIG. 4. Antibody production following a single injection of living, virulent tubercle bacilli. Two rabbits with residual foci of tuberculosis (crosses), and 2 previously healthy rabbits. Rabbit 16-20 injected 2 days later than the others (as indicated by arrow at top of chart).

these experiments are given in detail in Tables I and II. A definite phagocytosis-promoting power is present in the highest concentrations of serum from the first infection animals which is absent in the nor-

TABLE I.

Course of Tuberculous Infection (6th Week).

Details of experiment showing difference of response to virulent tubercle bacilli with respect to antibody formation. Three groups of rabbits are used: with residual foci of tuberculosis (reinfection), with primary infection (first infection), and normal animals, serving as controls.

	Serum dilutions					Sodium chloride control
	1:4	1:16	1:64	1:256	1:1024	
Reinfection Rabbit 16-14.						
Agglutination	++ to +	++ to +	++ to +	tr.	0	0
Resuspension	+++	++	++ to +	+	0	0
Cataphoresis	0.56	0.95	1.18	1.37	1.47	1.69
Phagocytosis	39	18	16	10	7	4
Reinfection Rabbit 16-20.						
Agglutination	++ to +	++ to +	++ to +	+	0	0
Resuspension	+++	++	+	+	0	–
Cataphoresis	0.82	0.97	1.22	1.08	1.46	1.65
Phagocytosis	27	16	19	13	10	4
Reinfection Rabbit 16-22.						
Agglutination	++ to +	++ to +	++ to +	+	sl. tr.	
Resuspension	++	++ to +	+	0	0	
Cataphoresis	0.78	0.93	1.23	1.68	1.67	1.54
Phagocytosis	26	18	14	7	7	
First Infection Rabbit 20-93.						
Agglutination	++ to +	+	+	sl. tr.		
Resuspension	+	sl. tr.	0	0		
Cataphoresis	1.19	1.21	1.33	1.46		1.74
Phagocytosis	14	10	4	3		
First Infection Rabbit 20-94.						
Agglutination	++ to +	+	tr.	sl. tr.		
Resuspension	tr.	0	0	0		
Cataphoresis	1.20	1.33	1.32	1.43		1.55
Phagocytosis	12	5	3	1		

TABLE I—*Continued.*

	Serum dilutions				Sodium chloride control
	1:4	1:16	1:64	1:256	
First Infection Rabbit 20-95.					
Agglutination	++ to +	++ to +	+	sl. tr.	
Resuspension	+	0	0	0	
Cataphoresis	1.20	1.38	1.36	1.47	1.71
Phagocytosis	12	10	3	1	
First Infection Rabbit 20-97.					
Agglutination	++ to +	++ to +	+ to tr.	sl. tr.	
Resuspension	++ to +	+ to tr.	0	0	
Cataphoresis	1.31	1.34	1.40	1.63	
Phagocytosis	10	5	1	3	
Normal Rabbit 21-28.					
Agglutination	+	+	+	sl. tr.	
Resuspension	0	0	0	0	
Cataphoresis	1.11	1.42	1.42	1.32	
Phagocytosis	2	4	1	3	
Normal Rabbit 21-56.					
Agglutination	++ to +	++ to +	tr.	0	
Resuspension	+	tr.	0	0	
Cataphoresis	1.15	1.35	1.43	1.54	
Phagocytosis	4	3	3	1	

mal sera. A very slight and indefinite difference in the same direction is suggested in the titers of the other tests. The loss of phagocytosis-promoting power of heated normal rabbit serum, which, however, is still able to alter the bacterial surfaces, has been discussed in the second paper of this series (8).

The sera of Rabbits 16-20, 16-22, 20-93, 20-94 and 20-95 were tested on June 6, in serial dilution for specificity against typhoid bacilli. They did not agglutinate the typhoid bacilli, which were shown to be agglutinated by anti-typhoid serum.

The autopsies in this group are in essential accord with those in Groups 1 and 2 and need not be given in detail. The reinfection

TABLE II.

Course of Tuberculous Infection (7th Week). See Legend of Table I.

The experiment here shown gives details of results obtained 1 week after the experiment presented in Table I.

	Serum dilutions						Sodium chloride control
	1:4	1:16	1:64	1:256	1:1024	1:4096	
Reinfection Rabbit 16-20.							
Agglutination	++ to +	++ to +	++ to +	+	0	0	0
Resuspension	+++	+++	++	++ to +	0	0	0
Cataphoresis	0.88	1.07	1.38	1.28	1 34	1.61	1.62
Phagocytosis	37	17	14	13	6	2	1
Reinfection Rabbit 16-22.							
Agglutination	++ to +	++ to +	++ to +	+	0	0	0
Resuspension	+++	+++	++	++ to +	sl. tr.	sl. tr.	0
Cataphoresis	0.67	0.90	1.28	1.50	1.52	1.59	1.35
Phagocytosis	37	21	19	10	5	1	2
First Infection Rabbit 20-93.							
Agglutination	+	++ to +	++ to +	+ to tr.	0		
Resuspension	+++	++ to +	sl. tr.	sl. tr.	sl. tr.		
Cataphoresis	1.32	1.27	1.58	1.51	1.42		1.57
Phagocytosis	15	7	4	6	3		
First Infection Rabbit 20-94.							
Agglutination	++ to +	++ to +	++ to +	+ to tr.	tr.		
Resuspension	++	+	sl. tr.	tr.			
Cataphoresis	1.26	1.28	1.36	1.36	1.41		1.34
Phagocytosis	10	4	2	1	3		
First Infection Rabbit 20-95.							
Agglutination	++	++ to +	+	+ to tr.	tr.		
Resuspension	++ to +	+	sl. tr.	sl. tr.	sl. tr.		
Cataphoresis	1.09	1.44	1.28	1.53	1.31		1.80
Phagocytosis	13	10	5	2	1		

TABLE II—*Continued.*

	Serum dilutions			
	1:4	1:16	1:64	1:256
Normal Rabbit 21-69.				
Agglutination	+	+	+	0
Resuspension	++ to +	+	sl. tr.	sl. tr.
Cataphoresis	0.91	1.27	1.35	1.39
Phagocytosis	6	2	5	
Normal Rabbit 21-73.				
Agglutination	+	++ to +	+	+ to tr.
Resuspension	+ to tr.	+ to tr.	sl. tr.	sl. tr.
Cataphoresis	1.32	1.30	1.36	1.42
Phagocytosis	4	2	4	
Normal Rabbit 21-75.				
Agglutination	++	++ to +	tr.	sl. tr.
Resuspension	++ to +	+	tr.	sl. tr.
Cataphoresis	1.09	1.23	1.39	1.38
Phagocytosis	2	3	0	
Normal Rabbit 21-77.				
Agglutination	+	+	+	+ to tr.
Resuspension	+	+ to tr.	sl. tr.	sl. tr.
Cataphoresis	1.05	1.28	1.36	1.53
Phagocytosis	5	3		

animals showed some residual tuberculosis in the lungs and kidneys, but no evidence of lesions due to the second infection. In the primarily infected rabbits, the infection ran its typical course.

DISCUSSION.

An augmented capacity of animals to produce antibodies against antigens to which they had previously been immunized was demonstrated by Cole in 1904.

Cole (9) immunized rabbits by intravenous injection of living typhoid bacilli and kept them until the resulting serum agglutinin titers had fallen to low values. Such rabbits on reinoculation with typhoid bacilli promptly produced antibodies in

considerable quantity in response to dosages which produced only negligible effects in normal rabbits. V. Liebermann and Acél (10) later demonstrated an augmented response in agglutinins and bactericidal antibodies in guinea pigs reinjected with typhoid bacilli. Dean and Webb (11) have reported the production of high titer antihorse sera in rabbits by two or more courses of injections at 2 or 3 months intervals. A single course of 8 or 10 or more injections gave sera of inferior titers. Schneider (12) revaccinated (with vaccinia virus) rabbits whose antivirus titers from the first vaccination had fallen to low values. Although revaccination led only to negligible visible symptoms, prompt and considerable increase in antivirus titers was demonstrated. Opie and Freund (13) found that the production of precipitin against crystalline egg albumin was accelerated and augmented in rabbits which had previously been immunized with crystalline egg albumin and had no precipitins in their blood at the time of reimmunization. It has also been found that antibody titers in previously vaccinated or infected men or animals may rise in response to heterologous antigens (14, 15, 16, 17), a phenomenon termed by Conradi and Bieling the "anamnestische Reaktion."

The most detailed account of these phenomena now available seems to be that of Bieling (18), who studied the production of antibodies in rabbits in response to injections of typhoid and of dysentery bacilli. Antibodies corresponding to the antigen first injected reappeared on later injection of either homologous or heterologous antigen; heterologous antibodies reappeared at once, homologous antibodies after a latent period.

One feature of the reactions described by Bieling suggests a relationship of these phenomena to that described by Lewis and Loomis (see below). According to Bieling (18) rabbits previously immunized with dysentery bacilli formed typhoid agglutinins in response to small injections of typhoid bacilli which were without effect in normal animals. At the same time the dysentery agglutinins again increase. The first specific immunization leaves behind therefore an increased capacity to respond to later injections which is non-specific.

It has been established, then, that an animal which has once produced antibodies in response to a specific antigen may reproduce these antibodies more quickly or in greater amount or both in response to a later injection of the homologous antigen, and may even produce such antibodies in response to injection of a heterologous antigen.[2] It seems clear that this phenomenon is at least a factor in the augmented

[2] In the present study the preliminary and reinfecting antigens used were essentially homologous. For although tubercle bacilli of human type were necessarily used for the preliminary infection, whereas reinfection was in most instances with bovine bacilli, human and bovine tubercle bacilli have repeatedly been found to be serologically indistinguishable. See (2) in which earlier references are cited.

production of antibodies against the tubercle bacillus which we have demonstrated in animals with residual tuberculous foci.

A phenomenon which may in part rest upon a common basis with the "anamnestic reaction" and which doubtless contributed to the augmented antibody production in our reinfection rabbits has been described by Lewis and Loomis (19). These authors found in tuberculous animals a greatly increased capacity for antibody production in response to a cellular and also a bacillary antigen. Other conditions than tuberculosis also provoked to some degree this heightened "allergic irritability."

In the present experiments there has been found a slight and slowly developing production of antibodies in first infection rabbits which showed (20) a moderate and slowly developing resistance to tubercle bacilli, and a prompt production of antibodies in quantity in reinfection animals found (20) to be highly resistant to the reinfecting tubercle bacilli.

The relation of antibodies to resistance in tuberculosis has recently been discussed by Opie (21). The present experiments suggest that an increased capacity to produce antibodies may be a factor in the heightened resistance of previously infected tuberculous animals. However, definite conclusions cannot be drawn at the present time.

The chief purpose for which these experiments were conducted was the testing under still another set of conditions of the relation between the changes brought about by sera in the surface properties and the phagocytosis of bacteria. The experimental error in these titrations was somewhat larger in proportion to the titers themselves than in the foregoing papers, but was not so large as to invalidate the interpretation of the results, even in the case of first infection animals. Within this error, complete correlation was again found between the effects of the sera in modifying the bacterial surface properties and in promoting phagocytosis, except in the case of heated normal sera; this exception had already been discussed. In experimental tuberculosis, as in the other conditions studied, whenever a serum has promoted phagocytosis it has produced in roughly corresponding degree the characteristic alterations in the bacterial surfaces,—and, with the exceptions noted,—whenever the surface changes have been produced phagocytosis has been promoted.

We believe that we have shown, then, that serum sensitization of acid-fast bacteria consists in or involves the combination with and deposition on the bacterial surface of a component or components which increase the cohesion, reduce the surface charge, alter the wetting properties and promote the phagocytosis of the bacteria. Further analysis of the process is in progress.

Throughout this work we have had able technical assistance from Mr. H. J. Henderson.

SUMMARY.

Rabbits infected intravenously with virulent mammalian tubercle bacilli have in a majority of cases developed circulating antibodies to a slight but appreciable degree. The increase in titer was detected in one group of rabbits within the 2nd or 3rd week, in others during the 2nd month of infection.

Rabbits with residual pulmonary foci, resulting from infection 6 months or more previously with human tubercle bacilli, on reinfection with bovine tubercle bacilli promptly developed circulating antibodies strikingly in excess of those found during the course of primary infection. Such antibodies were present 6 or 7 days after reinfection.

The changes in titer during tuberculous infection as detected by the bacterial surface reactions and by phagocytosis were again, within the experimental error, in quantitative correspondence. The loss of phagocytosis-promoting power in heated normal serum involves an exception to this correspondence between surface and phagocytosis effects. This exception has already been discussed in an earlier paper (8).

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TRANSMISSION OF RESPIRATORY ANAPHYLAXIS (ASTHMA) FROM MOTHER TO OFFSPRING.*

By BRET RATNER, M.D., and HELEN LEE GRUEHL.

(*From the Departments of Immunology and Pediatrics, University and Bellevue Hospital Medical College, New York University, New York.*)

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In the past it has been thought that nasal sensitization could be brought about only through the insufflation of solutions. Through work recently reported we have proved that nasal sensitization can be induced by the inhalation of a dry substance (horse dander). We have demonstrated[1] that when guinea pigs are subjected to an organic dust-laden atmosphere, they become sensitized, after a certain incubation period, to the substance which they have inhaled. We have termed this type of hypersensitiveness "respiratory anaphylaxis."

In a further series of studies[2] it was shown that a guinea pig sensitized by parenteral injection prior to or during pregnancy will sensitize her offspring in utero—passively or actively—and that this mechanism is traceable to the permeability of the placenta.

It has now become of interest to determine whether a pregnant animal made sensitive by inhalation, can transmit sensitizing substances to her fetus in utero.

EXPERIMENTS.

For these experiments, mature female guinea pigs were kept in our animal house for a number of weeks. In no instance was any evidence

* Preliminary report, *Proc. Soc. Exper. Biol. & Med.*, 1928, xxvi, 8. This work is being carried on under "The Crane Research Fund for the Study of Allergic Diseases in Children."

[1] Ratner, B., Jackson, H. C., and Gruehl, H. L., *Proc. Soc. Exper. Biol. & Med.*, 1925, xxiii, 16, 17; *Am. J. Dis. Child.*, 1927 xxxiv, 23. Ratner, B., and Gruehl, H. L., *Proc. Soc. Exper. Biol. & Med.*, 1928, xxv, 661.

[2] Ratner, B., Jackson, H. C., and Gruehl, H. L., *Proc. Soc. Exper. Biol. & Med.*, 1926, xxiii, 327; *J. Immunol.*, 1927, xiv, 249, 267, 275, 291, 303.

of dyspnea observed. After this period of observation, the animals were placed in cages with normal males for mating and then exposed to horse dander dust—in a manner previously described[3]—during various periods of time. In certain instances conception occurred prior to the period of sensitization. Hence it came about that we had mothers which had been sensitized to horse dander dust either before or during pregnancy. After varying intervals of time, these pregnant animals were again placed in contact with horse dander dust and in the majority of instances they manifested definite respiratory anaphylaxis (asthma).

Soon after birth, the offspring of these mothers were brought into contact with horse dander dust and their reactions on first contact with this substance noted. 1 hour later both the mothers and their offspring were given an intravenous injection of an alkaline extract of this horse dander,[4] in order to test whether such respiratory disturbances as had been observed were anaphylactic in nature. The details of our experiments are given in the following protocols.

PROTOCOLS.

Family 1.

Female 1030 (510 gm.) was placed in the inhalation chamber and exposed to the horse dander dust for a total of 9 hours between Nov. 21, 1927 and Dec. 1, 1927; in no instance was there any evidence of dyspnea. On Feb. 16, 1928, or 2½ months later, she was placed in the inhalation chamber and showed severe convulsions and marked dyspnea—definite respiratory anaphylaxis. At varying intervals of time she was again brought into contact with this dust and exhibited definite symptoms of respiratory anaphylaxis. On May 10, 1928 she gave birth to 2 offspring.

Offspring 1454, 1455 (90, 100 gm.), when 5 days old, on May 15, 1928, each received an intravenous injection of 0.5 cc. dander extract and both died in typical anaphylactic shock.

Mother on the same day was given 0.5 cc. dander extract intravenously and also died in typical anaphylactic shock with lungs typically ballooned.

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[3] Ratner, B., Jackson, H. C., and Gruehl, H. L., *Am. J. Dis. Child.*, 1927, xxxiv, 23.

[4] Ratner, B., Jackson, H. C., and Gruehl, H. L., *Am. J. Dis. Child.*, 1927, xxxiv, 23.

Family 2.

Female 1311 (640 gm.) was sensitized by exposure to horse dander dust for 8 hours from Jan. 20, 1928 to Jan. 28, 1928. On Feb. 13, 1928, 24 days after her initial exposure, she gave birth to 1 pig.

Offspring 1344 when 4 days old was placed in the inhalation chamber and showed definite evidences of moderate respiratory anaphylaxis.

Mother.—This guinea pig again became pregnant shortly after the previous confinement and at varying intervals, on being placed in the inhalation chamber, showed evidences of marked respiratory anaphylaxis. The second litter of 3 offspring was born on July 11, 1928.

Offspring—second litter—1516, 1517, 1518 (65, 70, 85 gm.) were placed in the inhalation chamber when 1 day old and all had marked respiratory anaphylaxis. This respiratory anaphylaxis was much more severe than that exhibited by the offspring of the first confinement. On the same day each received an intravenous injection of 0.3 cc. dander extract and all died in typical anaphylactic shock.

Mother (795 gm.) on the same day manifested marked respiratory anaphylaxis when placed in the inhalation chamber and on intravenous injection of 0.5 cc. dander extract showed profound anaphylaxis, dyspnea and collapse with final recovery.

Family 3.

Female 1509 (745 gm.) was exposed to horse dander dust for 6 hours in all from May 31, 1928 to June 6, 1928 and showed no signs of dyspnea during the exposures. 3 weeks after the initial exposure, on June 20, 1928, she was again placed in the inhalation chamber and showed marked respiratory anaphylaxis. This respiratory anaphylaxis was manifested at another contact with horse dander. On July 26, 1928, a little less than 2 months after the initial sensitizing exposure, the animal gave birth to 2 offspring.

Offspring 1594 and 1595 (80 gm. each) when 1 day old were placed in the inhalation chamber and manifested marked symptoms of respiratory anaphylaxis. On the same day each received 0.3 cc. dander extract intravenously following which both died in typical anaphylactic shock.

Mother on the same day, when placed in the inhalation chamber, exhibited moderate respiratory anaphylaxis and on intravenous injection of 0.5 cc. dander extract showed unmistakable signs of anaphylaxis—collapse, dyspnea, suffusion of the eyes, with final recovery.

Families 4 and 5 gave similar results.

Family 6.

Female 1217 (550 gm.) was sensitized in the inhalation chamber for 9 hours from Nov. 21, 1927 to Dec. 1, 1927 with no evidences of dyspnea. When again placed in the inhalation chamber on Feb. 16, 1928 she showed marked respiratory

anaphylaxis. This respiratory anaphylaxis was repeated at various intervals whenever the animal was placed in the inhalation chamber. On Sept. 3, 1928 she gave birth to 2 offspring.

Offspring 1626, 1627 (125, 120 gm.) when 10 days old, on Sept. 13, 1928, were placed in the inhalation chamber for the first time and showed marked evidences of respiratory anaphylaxis. Offspring 1626 gave a very profound reaction. It recovered, however, and after an intravenous injection of 0.3 cc. dander extract it showed only marked anaphylaxis with recovery. On the other hand Offspring 1627, which was not so profoundly affected in the inhalation chamber, died in acute anaphylactic shock after an intravenous injection of 0.3 cc. dander extract.

Mother on the same day showed moderate respiratory anaphylaxis in the inhalation chamber and after an intravenous injection of 0.5 cc. dander extract exhibited definite anaphylaxis with recovery.

Family 7.

Female 1466 (740 gm.) was sensitized in the inhalation chamber for 3½ hours from May 24, 1928 to May 29, 1928 and showed no dyspnea at all. When again placed in the inhalation chamber on June 13, 1928, 3 weeks after the initial exposure, she had no evident reaction. On June 28, 1928, she gave birth to 2 offspring.

Offspring 1512, 1513 (60, 65 gm.) when 12 hours old were placed in the inhalation chamber and showed profound respiratory anaphylaxis. Intravenous injection of 0.3 cc. dander extract produced death in Offspring 1512 and marked anaphylaxis—convulsions, collapse, dyspnea—with recovery in Offspring 1513.

Mother.—It is interesting that the mother when placed in the inhalation chamber on the same day was still negative and showed only slight symptoms of anaphylaxis after an intravenous injection of 0.5 cc. dander extract.

Family 8.

Female 1467 (610 gm.) was placed in the inhalation chamber for 3½ hours from May 24, 1928 to May 29, 1928 and showed no signs of respiratory anaphylaxis. On June 13, 1928, after an incubation period of 3 weeks, when again placed in the inhalation chamber, she was negative. 2 weeks after this, however, when placed in the inhalation chamber she demonstrated marked respiratory anaphylaxis. On July 21, 1928, a little less than 2 months after the beginning of sensitization, she gave birth to 3 offspring.

Offspring 1583, 1584, 1585 (50, 85, 75 gm.) when 3 days old, on July 24, 1928 showed moderate dyspnea when placed in the inhalation chamber and moderate signs of anaphylaxis after an intravenous injection of 0.3 cc. dander extract.

Mother on the same day in the inhalation chamber and after an intravenous injection of 0.7 cc. dander extract, showed the same moderate symptoms as did the offspring.

Families 9, 10, 11 and 12 gave similar results.

Family 13.

Female 1460 (700 gm.) was exposed to dander in the inhalation chamber for ½ hour on May 23, 1928 and ½ hour on May 24, 1928 and showed no dyspnea. This animal gave birth to 2 offspring on May 25, 1928, the 2nd day after the initial exposure.

Offspring 1482, 1483 (215, 210 gm.) when 27 days old, on June 21, 1928, were both put in the inhalation chamber. Offspring 1482 showed marked respiratory anaphylaxis and after intravenous injection of 0.5 cc. dander extract died in typical anaphylactic shock. Offspring 1483 was practically negative in the cage but after an intravenous injection of 0.5 cc. dander extract manifested moderate anaphylaxis with recovery.

Mother on the same day—28 days after her initial exposure to dander—was placed in the inhalation chamber and was negative. On the same day she showed definite anaphylaxis with recovery after an intravenous injection of 0.5 cc. dander extract.

Family 14.

Female 1265 (400 gm.) was exposed to dander in the inhalation chamber for 11 hours from Dec. 22, 1927 to Jan. 13, 1928 with no signs of dyspnea. On Jan. 16, 1928, 25 days after the initial exposure to dander, she gave birth to 2 offspring.

Offspring 1308, 1309 (190, 150 gm.) on Feb. 7, 1928, when 22 days old, were not placed in the inhalation chamber but were each given an intravenous injection of 0.5 cc. dander extract. Offspring 1308 was practically negative and Offspring 1309 showed only suffusion of the eyes.

Mother on the same day, however, when given an intravenous injection of 0.5 cc. dander extract had convulsions, collapse, marked dyspnea and died in typical anaphylactic shock after collapse for about ¾ hour.

Family 15.

Female 1173 (540 gm.) was exposed to dander in the inhalation chamber for 10 hours from Nov. 7, 1927 to Nov. 19, 1927 with no signs of dyspnea. On Feb. 16, 1928, 3¼ months after the initial exposure, when again placed in the inhalation chamber, this animal showed definite respiratory anaphylaxis. She was placed in the inhalation chamber at 2-week intervals throughout her pregnancy and each time exhibited marked respiratory anaphylaxis. On July 13, 1928 this animal gave birth to 1 offspring.

Offspring 1541 (130 gm.) on July 19, 1928, when 6 days old, showed moderate dyspnea in the inhalation chamber and after intravenous injection of 0.3 cc. dander extract had marked anaphylaxis with collapse and final recovery.

Mother on the same day was negative in the inhalation chamber and also after intravenous injection of 0.5 cc. dander extract.

Family 16.

Female 1461 (580 gm.) was exposed to dander in the inhalation chamber for 4 hours from May 23, 1928 to May 29, 1928, with no evidence of dyspnea. On June 8, 1928, 16 days after the initial contact with dander, she gave birth to 3 offspring.

Offspring 1493, 1494, 1495 (120, 130, 130 gm.) when 13 days old, on June 21, 1928, were placed in the inhalation chamber for the first time and were negative. Each animal received an intravenous injection of 0.5 cc. dander extract on the same day; Offspring 1493 was negative, Offspring 1494 and 1495 each showed moderate anaphylaxis with recovery.

Mother on the same day was placed in the inhalation chamber and had typical respiratory anaphylaxis. After an intravenous injection of 0.5 cc. dander extract she showed typical anaphylaxis with recovery.

Family 17.

Female 1408 (840 gm.) was exposed to dry horse dander for 6 hours from Mar. 15, 1928 to Mar. 21, 1928 and showed no signs of dyspnea. This sensitization was prior to pregnancy. This animal was placed in the inhalation chamber at 2-week intervals and was negative until about 1½ months before confinement when she began to develop symptoms of moderate respiratory anaphylaxis. On July 30, 1928, 4 offspring were born.

Offspring 1596, 1597, 1598, 1599 (80, 60, 75, 70 gm.) when 1 day old, on July 31, 1928, were placed in the inhalation chamber and all showed only suggestive symptoms of respiratory anaphylaxis. On intravenous injection of 0.3 cc. dander extract each again showed only suggestive symptoms.

Mother on the same day when placed in the inhalation chamber and also after intravenous injection of 0.5 cc. dander extract demonstrated only suggestive symptoms.

Family 18.

Female 1463 (600 gm.) was placed in the inhalation chamber for 3½ hours from May 24, 1928 to May 29, 1928 with no symptoms of dyspnea. On June 18, 1928, 25 days after the initial exposure, when again placed in the inhalation chamber she showed signs of moderate dyspnea but when subsequently placed in the inhalation chamber at various intervals, there were no signs of dyspnea. On July 24, 1928, 2 months after the beginning of sensitization, 3 offspring were born.

Offspring 1586, 1587, 1588 (75, 70, 75 gm.) were placed in the inhalation chamber when 2 days old, on July 26, 1928, and gave a slight to negative reaction. On the same day an intravenous injection of 0.3 cc. dander extract brought forth no anaphylactic reaction.

Mother on the same day showed no symptoms of anaphylaxis after exposure to dander and none after intravenous injection of 0.6 cc. dander extract.

Family 19.

Female 1469 (520 gm.) was placed in the inhalation chamber for 3½ hours from May 24, 1928 to May 29, 1928 and showed no dyspnea. On June 7, 1928 she gave birth to 2 offspring.

Offspring 1491, 1492 (55, 35 gm.) were placed in the inhalation chamber when 1 day old, on June 8, 1928, and were both negative. They showed no signs of anaphylaxis after an intravenous injection of 0.3 cc. dander extract.

Mother was negative when placed in the inhalation chamber on the same day and also after an intravenous injection of 1 cc. dander extract.

Families 20, 21 and 22 gave similar results.

Family 23.

Female 1473 (510 gm.) was placed in the inhalation chamber for 6 hours from May 31, 1928 to June 6, 1928 with no signs of dyspnea. On June 20, 1928, 20 days after the initial contact, she was again placed in the inhalation chamber and was negative. On June 23, 1928, 2 offspring were born.

Offspring 1494, 1495 (60, 55 gm.) were placed in the inhalation chamber on June 26, 1928, when 3 days old, and were negative. They were also negative after an intravenous injection of 0.5 cc. dander extract.

Mother on the same day was negative in the inhalation chamber and after intravenous injection of 0.5 cc. dander extract.

Families 24, 25 and 26 gave similar results.

ANALYSIS OF PROTOCOLS.

As controls for these experiments we had 11 new-born animals which manifested no signs of dyspnea when exposed to dander in the inhalation chamber. These animals were injected intravenously with horse dander solution and manifested no reaction after the injection. This indicates that only specifically sensitized new-born animals will evidence anaphylactic symptoms. The adult guinea pigs have been shown to be non-sensitive because none of them manifested any signs of dyspnea during the period of sensitization.

In Family 1 we have an animal sensitized by inhalation of dry horse dander before pregnancy which, at various times throughout her pregnancy, was brought into contact with this dust and manifested severe respiratory anaphylaxis (asthma). When her offspring were 5 days old and were given an intravenous injection of dander extract, they died in acute anaphylactic shock. These 2 offspring demonstrate that an animal sensitized merely by inhalation and which has had

TABLE 1.

Summary of Protocols.

	Family	Mother			Offspring		
		Respiratory anaphylaxis before birth of offspring	Respiratory anaphylaxis after birth of offspring	Intravenous injection	No.	Respiratory anaphylaxis	Intravenous injection
1	1030	++	Not done	+++	1454	Not done	+++
					1455	Not done	+++
2	1311	Not done	Not done	Not done	1344	+	Not done
		++	++	++	1516	++	+++
					1517	++	+++
					1518	++	+++
3	1509	++	+	++	1594	++	+++
					1595	++	+++
4	1476	++	−	++	1606	++	+++
5	1410	++	+	++	1489	++	+++
					1490	++	+++
6	1217	++	+	++	1626	++	++
					1627	++	+++
7	1466	−	−	+−	1512	++	+++
					1513	++	++
8	1467	++	+	+	1583	+	+
					1584	+	+
					1585	+	+
9	1215	++	++	++	1580	++	++
					1581	++	++
					1582	++	++
10	1481	++	+	+	1631	+	++
					1632	+	++
11	1480	++	−	+	1623	+	+
					1624	+	+
					1625	+	+
12	1479	Not done	+	++	1496	+	+
					1497	+	+

− No reaction.
+− Slight symptoms of anaphylaxis.
\+ Moderate symptoms of anaphylaxis.
++ Marked symptoms of anaphylaxis.
+++ Typical anaphylactic death with distended lungs.

TABLE I—*Concluded.*

	Family	Mother			Offspring		
		Respiratory anaphylaxis before birth of offspring	Respiratory anaphylaxis after birth of offspring	Intravenous injection	No.	Respiratory anaphylaxis	Intravenous injection
13	1460	Not done	−	++	1482	++	+++
					1483	−	+
14	1265	Not done	Not done	+++	1308	Not done	+−
					1309	Not done	+−
15	1173	++	−	−	1541	+	++
16	1461	Not done	++	++	1493	−	−
					1494	−	+
					1495	−	+
17	1408	+	+−	+−	1596	+−	+−
					1597	+−	+−
					1598	+−	+−
					1599	+−	+−
18	1463	+	−	−	1586	+−	−
					1587	+−	−
					1588	+−	−
19	1469	Not done	−	−	1491	−	−
					1492	−	−
20	1310	Not done	−	Not done	1336	−	Not done
21	1266	Not done	++	Not done	1335	−	Not done
22	1475	Not done	−	−	1484	−	−
					1485	−	−
23	1473	−	−	−	1494	−	−
					1495	−	−
24	1333	−	−	Not done	1452	Not done	−
					1453	Not done	−
25	1468	−	−	−	1514	−	−
					1515	+	−
26	1270	Not done	Not done	−	1334	−	−

respiratory anaphylaxis during pregnancy can give birth to offspring which are sensitive to the same substance.

Families 2, 3, 4 and 5 demonstrate the transmission of respiratory anaphylaxis from mother to offspring, that is, the offspring, when exposed to horse dander for the first time gave evidence of respiratory anaphylaxis.

Families 6, 7, 13 and 16 also demonstrate the transmission of respiratory anaphylaxis from a mother to her offspring but show in all instances a varying degree of sensitization in the offspring. In 3 families 1 offspring died in anaphylactic shock while the other offspring showed anaphylaxis with recovery. In Family 16, 1 offspring was entirely negative while the other 2 showed moderate anaphylaxis. In this last case the mother was strongly anaphylactic.

In Families 8, 9, 10, 11 and 12 we have instances of respiratory anaphylaxis to a more moderate degree in both mother and offspring than has been evidenced in the above families.

Family 13 demonstrates active sensitization of a fetus in utero.[5] The mother was exposed to the horse dander dust for 1 hour only. 2 days after her initial contact she gave birth to 2 offspring. These offspring were permitted to live in a normal environment for 27 days and were then exposed to dander in the inhalation chamber for the first time. They showed marked respiratory anaphylaxis (asthma) in the inhalation chamber and 1 died when given an intravenous injection of dander extract. Here we have an instance of the transfer not of antibodies from a mother which has suffered from respiratory anaphylaxis during pregnancy, but the passage of antigen through the upper respiratory tract into the mother's circulation and thence into the circulation of the fetus. In the fetal blood this antigen brings about the development of active sensitization after a suitable incubation period has elapsed.

In Families 14 and 16 we have instances in which the mother was profoundly sensitive and the offspring were only moderately sensitive. The only symptoms of anaphylaxis shown in Family 14 was that of suffusion of both eyes of 1 offspring. The symptoms of suffusion were also shown by Animals 1509 and 1479.[6]

[5] Ratner, B., Jackson, H. C., and Gruehl, H. L., *J. Immunol.*, 1927, xiv, 303.

[6] Ratner, B., Jackson, H. C., and Gruehl, H. L., *Proc. Soc. Exper. Biol. & Med.* 1927, xxiv, 444.

Families 7 and 15 show interesting instances of the sensitization apparently having worn off in the mother whereas there is still a moderate anaphylactic sensitization present in the offspring.

In Families 17 and 18 we have only an extremely moderate degree of respiratory anaphylaxis in the mother and in the offspring. In Family 17 where the sensitization was carried on over a long period of time the transmission of respiratory anaphylaxis was no more marked than in Family 18.

In Families 19, 20, 21 and 22 there has been an absence of transmission of respiratory anaphylaxis probably because of the short period of exposure of the mother. In these cases the mothers themselves were not sensitive.

In Families 23, 24, 25 and 26, on the other hand, we have instances of a sufficiently long period of sensitization in the mother with neither the mothers nor the offspring showing any sensitization.

In Family 2 we have an instance of two litters born to the same animal. In the case of the first litter the time between the beginning of sensitization and confinement of the mother was only 23 days and the offspring showed only moderate anaphylaxis. In that of the second litter, born after the mother had had many attacks of respiratory anaphylaxis (asthma), throughout her pregnancy, the offspring were profoundly sensitive and all died after an intravenous injection of dander extract.

DISCUSSION.

Normal guinea pigs, whether they be pregnant mothers or offspring only a few days old, will not manifest dyspnea when exposed to a dust to which they are not sensitive. This has been demonstrated by our many experiments.

In order to prove the anaphylactic character of the symptoms and to rule out pneumonia or any other pathologic condition of the guinea pig which might simulate the dyspnea found in respiratory anaphylaxis,[7] the animals received an intravenous injection of an extract of the dust on the same day that they demonstrated this syndrome and in all cases necropsies were performed.

[7] Ratner, B., Jackson, H. C., and Gruehl, H. L., *Am. J. Dis. Child.*, 1927, xxxiv, 23.

It may be of interest to comment on the fact that in 26 families studied we have no single instance of miscarriage during the attacks of respiratory anaphylaxis (asthma) even in the latter period of pregnancy.

Analysis of our protocols demonstrates that a mother guinea pig which has manifested respiratory anaphylaxis during pregnancy may passively[8] transmit the state of hypersensitivity to her offspring in utero. In one instance this was brought about by an active sensitization in utero. When the offspring are first brought into contact with a dust to which the mother is sensitive, they exhibit respiratory anaphylaxis (asthma) of the same sort she showed.

There were certain variations in the transmission which call for further discussion.

Because of the nature of the experiments, it has been impossible to sensitize and expose these mothers in a uniform manner. For this reason—as is evident in Table I—instances occurred in which sensitization was firmly established in the mothers and was transmitted, other instances in which the mothers were more profoundly sensitized than the offspring or conversely in which the offspring had a higher degree of sensitization than the mother; instances in which the sensitization established was of a moderate grade; and finally instances in which there was no sensitization of either mother or offspring. Several of the families in this last group undoubtedly had too short a period of exposure for the establishment of sensitization, while others, although the period was long enough apparently, were not sensitized.

In Family 2 we have an interesting example of the influence of the period upon the results. The offspring of the first litter was only moderately sensitive whereas those of the second litter, after the mother had shown profound respiratory anaphylaxis throughout her pregnancy, were sensitive and died in typical anaphylactic shock.

We were fortunate in having 1 family in which the mother was exposed for only 2 days before confinement. The offspring in this instance could not have received sensitizing antibodies but must have developed an active sensitization from antigen transmitted in utero. This mechanism has been demonstrated by us in a previous study.

[8] Ratner, B., Jackson, H. C., and Gruehl, H. L., *J. Immunol.*, 1927, xiv, 291.

In our previous work[9] it was shown that guinea pigs injected prior to or during pregnancy might give birth to sensitive offspring; and in another study[10] we presented instances of human mothers eating excessively of certain foods during pregnancy who gave birth to children sensitive to those foods.

We believe the experiments presented in this paper demonstrate a manner in which the human offspring of an asthmatic mother might be sensitized. Furthermore, the experiments indicate that mothers with the asthma of anaphylaxis may not always give birth to asthmatic children and on the other hand that mothers, themselves not asthmatic, may transmit sensitizing antigen to their offspring.

CONCLUSIONS.

1. A further method is offered whereby sensitization in utero may be established.

2. Respiratory anaphylaxis—induced in a pregnant guinea pig by the inhalation of a dry antigenic dust—can thus be transmitted from mother to offspring.

3. A guinea pig thus sensitized in utero, when brought into contact for the first time with an anaphylactogenic dust to which the mother was sensitized, will manifest respiratory anaphylaxis.

4. The transmission of this hypersensitiveness may be brought about passively through the transmission of sensitizing antibodies.

5. A fetus may be actively sensitized in utero by a mother which has inhaled the antigenic dust and has not herself been sensitive at the time of birth.

6. This state of hypersensitiveness may be transmitted in varying degrees of intensity and when 2 or more offspring are born in the same litter, they may, in some instances, be sensitized to an equal degree and sometimes to different degrees.

7. This state of hypersensitiveness can be transmitted through more than 1 litter.

8. All animals cannot be made hypersensitive.

[9] Ratner, B., Jackson, H. C., and Gruehl, H. L., *J. Immunol.*, 1927, xiv, 249, 267, 275, 291, 303.

[10] Ratner, B., *Am. J. Dis. Child.*, 1928, xxxvi, 277.

A "SOLUBLE SPECIFIC SUBSTANCE" DERIVED FROM GUM ARABIC.*

By MICHAEL HEIDELBERGER, Ph.D., OSWALD T. AVERY, M.D., and WALTHER F. GOEBEL, Ph.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

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The unforeseen identification of the so-called "soluble specific substances" (1) of Pneumococcus as polysaccharides (2) has led to an accumulation of evidence that analogous sugar derivatives play an important part in the immunological relationships of micro-organisms of the most diverse types (3). Thus, specifically reacting substances with the properties of carbohydrates have also been isolated from the Friedländer bacillus, the tubercle bacillus, the typhoid-colon group, and yeast; and evidence has been obtained of the existence of similar substances in Streptococcus, the anthrax bacillus, and other pathogenic microbes. This wide distribution of specifically reacting polysaccharides made it seem not improbable that there might occur among higher plant forms other sugar derivatives with specific properties. A number of water-soluble gums of plant origin were therefore tested against anti-pneumococcus sera of Types I, II, and III, and of these gums several were found to give the precipitin reaction. Since occasional samples of gum arabic (gum acacia) precipitated Type II (and Type III) antiserum at as high a dilution as 1:25,000 (*cf.* Table I, 47_2), but did not precipitate normal horse serum or Type I antiserum, this gum was chosen for further study.

It was soon found that the ordinary methods of fractional precipitation from neutral, acid, or alkaline solutions yielded products differing

* An abstract of this paper was presented at the Annual Meeting of the American Chemical Society in Philadelphia, in Sept., 1926. The paper itself was submitted for publication at the same time, but was withdrawn until more positive analytical data on the calcium aldobionate were available. In the meantime Cretcher and Butler have also published a note (*Science*, Aug. 3, 1928) indicating the presence of an aldobionic acid among the products of hydrolysis of gum arabic and have a paper in press giving further details.

little in their specific reactivity. Since it was known that the soluble specific substance of Pneumococcus was comparatively resistant to strong acid in the cold (*2b*, p. 305), fractional hydrolysis of this type was resorted to in the hope that the non-specific portions of the gum would prove the more easily hydrolyzed. This appeared to be the case, as the polysaccharide fraction recovered was found to possess a degree of specificity comparable with that of the bacterial specific substances (*cf.* Table I, 47_{10BIV}, 56). From the fact that 50 per cent of material 100 to 150 times as active as the original gum was recovered it is evident, however, that the process involves more than a mere hydrolysis of accompanying inert material. This point will be taken up more fully in the discussion.

The gum arabic purified in this way resembled in its physical properties the arabic acid so frequently described as the principal constituent of the gum (4). On hydrolysis, however, it yielded mainly galactose and an acid fraction consisting of at least 2 substances. Investigation of these acids has indicated that they are possibly disaccharide (aldobionic) acids of the type recently described as the principal product of hydrolysis of the soluble specific substance of Type III pneumococcus (*3a* and *3b*). Such a relationship would be of considerable theoretical interest in a study of the chemistry of bacterial specificity. The large quantities of starting material available should facilitate the investigation of aldobionic acids to an extent impossible in the case of the polysaccharides elaborated by pathogenic bacteria.

EXPERIMENTAL.

1. Isolation of Reactive Material from the Original Gum.

200 gm. of Squibb's powdered gum acacia were dusted slowly into 1 liter of chilled 1:1 hydrochloric acid. The mixture was stirred until solution was complete and allowed to stand at room temperature for 2 days. A small amount of dark, insoluble material was centrifuged off and the clear liquid precipitated with about 3 volumes of chilled alcohol. After the gum had settled the supernatant was poured off and the precipitate macerated with fresh alcohol. After several hours this was decanted and the gum was dissolved in water, centrifuged if necessary, and reprecipitated with redistilled acetone in the cold. After several hours the precipitate was stirred with fresh acetone, ground up under acetone when thoroughly hardened, filtered, washed with acetone until free from hydrochloric acid, and dried *in vacuo* over calcium chloride and crushed sodium hydroxide.

The yield was 70 to 80 gm. This product (Table I, 56) corresponded closely to that obtained in 97.5 gm. yield by a single acid treatment of 24 hours (47_{10}) and to preparation 47_{10BIV} obtained in 64 gm. yield by 2 single acid treatments of 24 hours each. It was sometimes necessary to redissolve the gum in water a second time and reprecipitate with acetone in order to remove all chlorine ion.

The product so obtained still contained about 0.3 per cent of nitrogen, or the entire amount in the original gum. 60 gm. of preparation 47_{10BIV} were therefore dissolved in 300 cc. of water and stirred ½ hour with 5 cc. of 30 per cent sodium nitrite solution and 25 cc. of acetic acid. About 1.5 volumes of acetic acid were then added, precipitating most of the gum. After 2 hours the deposit was drained, dissolved in about 200 cc. of water, and reprecipitated with acetic acid. It was finally treated with successive portions of redistilled alcohol and acetone, ground up, washed thoroughly with acetone, and dried as before. The yield was 42.3 gm. This product (Table I, 51E) contained less than 0.1 per cent of nitrogen and reacted with Type II anti-pneumococcus serum at a dilution of 1:5,000,000. It also precipitated Type III anti-pneumococcus serum. The fraction of the gum not thrown down by the acetic acid resembled the precipitated portion in all its properties, but it contained 0.5 per cent of nitrogen and was somewhat less reactive with Type II serum.

The purified gum is a white powder, readily soluble in water. It possesses marked acidic properties and rotates the plane of polarized light weakly to the left, somewhat more strongly on neutralization. It gives a positive naphthoresorcin test. When hydrolyzed it yields 68 per cent of reducing sugars, calculated as glucose, but since about one-third of the products of hydrolysis appear to be disaccharide or polysaccharide acids (see below), the actual yield of reducing sugars is higher. The pentose content, 19 per cent, calculated from the yield of furfural on distillation with hydrochloric acid,[1] is about one-half that of the original gum, so that much of the portion hydrolyzed in the method of preparation consisted of pentose or pentosan. Part of the remaining material which reacts as pentose is accounted for by the sugar acid fraction. The principal hexose component of the purified gum is galactose.

[1] A modification of Pervier and Gortner's method (5) was used. Instead of titrating at an acidity of 4 per cent and plotting the end-point with the aid of a bromine electrode, galvanometer, and stop-watch, it was found simpler to use an outside spot indicator of starch iodide solution. At an acidity of 3 per cent, the end-point is taken as the first burette reading at which a spot test is still obtained after 2 minutes. Large drops should be withdrawn for the test.

2. Attempts at Further Purification of the Specific Fraction.

The specific fraction of the gum is incompletely precipitated by barium hydroxide in large excess. The precipitate soon turns yellow, and as will be seen from Table I the recovered gum (51B) shows practically the same properties as the original material.

Uranyl nitrate also precipitates the gum incompletely when the excess acid is neutralized, but in this case also no purification results.

Partial adsorption of specific gum on "Type C" aluminium hydroxide (6) resulted only in a product with the properties of the starting material (Table I, 54D).

Fractionation of the specific product with hydrochloric acid and alcohol gave 3 portions with practically identical properties.

3. Precipitation of the Specific Gum by Means of Type II Pneumococcus Antibodies.

An attempt was made to determine whether the specific gum could be precipitated by Type II pneumococcus antiserum and recovered from the precipitate. This was of importance not only in establishing the polysaccharide as actually analogous or not to the specific polysaccharides of bacterial origin, but also in determining whether the reaction with Type III antiserum was caused by an accompanying impurity or was an inherent property of the specific polysaccharide itself. Thus, an accompanying substance which did not precipitate Type II serum, but yielded a precipitate with Type III serum, should be largely eliminated in effecting a specific precipitation with Type II serum.

3 liters of Type II pneumococcus antibody solution, prepared essentially by Felton's method (7), were precipitated by a slight excess of neutralized 1:1000 saline solution of preparation 47_{10}. The amount of precipitate was small, and only 0.07 gm. of specific gum was recovered by the method already described in detail in the case of the soluble specific substance of Type II pneumococcus (2*c*, p. 737).

Except for an unavoidably high nitrogen content (0.6 per cent) and a higher specific reactivity toward both sera the product (Table I, 47_{10}A) resembled the starting material in its analytical and physical properties. It still gave a precipitate with barium hydroxide in excess, gave the brown red color characteristic of galactans with orcin

TABLE I.

Properties of Gum Arabic and Derived Specific Fractions.

Preparation No.	$[\alpha]_D$		Acid equivalent	Nitrogen	Reducing sugars on hydrolysis		Highest dilution precipitating pneumococcus antiserum		Ash	
	Free acid	Na salt			Total	Pentose	Type II	Type III		
				per cent	*per cent*	*per cent*			*per cent*	
47_2		−31.0°		0.35	77.4	40	25,000	25,000	1.4	Squibb's gum acacia
47_{10}	−7.5°	−8.8°	1006			15	3,000,000*	1,000,000	0.3	Single acid treatment, 24 hours
$47_{10\text{-BIV}}$	−12.0°	−16.0°	906			19	4,000,000	+	0.03	Two acid treatments
$47_{10}A$		−20.0°	665	0.6	67.3		8,000,000	2,000,000	0.3	Recovered from immune precipitate
51B	−12.0°			0.15			4,000,000	+	0.1	From $Ba(OH)_2$ precipitate
51E	−10.7°	−14.3°	856	0.08	68.0	18	5,000,000	+	0.06	47_{10BIV}, treated with HNO_2 and precipitated with acetic acid
54D	−11.5°	−16.6°	723	0.2	68.5	18	4,000,000	+	0.03	Adsorbed on $Al(OH)_3$
56	−7.5°	−12.5°	866	0.33	68.0	19	3,000,000†	+	0.13	Single acid treatment, 48 hours

* There is apparently relatively little antibody to the specific gum in Type II and Type III antisera. In the former case the precipitate is a transparent jelly, and the tubes containing the higher dilutions must be centrifuged in order to render the deposit more compact and more easily visible. The Type III precipitate is loose and flocculent until centrifuged.

† Highest dilution tested.

and hydrochloric acid, and showed a positive naphthoresorcin test. Not only did it react at a dilution of 1:8,000,000 with Type II antipneumococcus serum, but also at a dilution of 1:2,000,000 with Type

III serum, indicating that it is actually the same substance which precipitates with both sera.

While this experiment seemed fairly conclusive in establishing the purified gum as a true "soluble specific substance," it remained possible that the polysaccharide reacted with some other constituent of the serum than the pneumococcus antibodies themselves.

Accordingly a portion of the Type II antiserum was absorbed by means of a saline suspension of heat-killed Type II pneumococci. Another portion of the serum was treated with small amounts of a 1:10,000 solution of the Type II soluble

TABLE II.

Dilution of preparation 47_{10BIV}	Type II serum unabsorbed	Type II serum absorbed with Pneumococcus II	Type II serum absorbed with soluble specific substance II	Type II antibody solution absorbed with specific gum arabic
1:1,000	++++	−	−	
1:5,000	+++	−	−	
1:25,000	+++	−	−	
1:250,000	++	−	−	
1:1,000,000	−	−	−	
Saline	−	−	−	
Dilution of Type II soluble specific substance, 1:20,000			−	++++
Agglutination of Type II pneumococci			−	++++

Dilution of serum, 2:3. Tubes were not centrifuged. After centrifugation the 1:1,000,000 dilution reacted + in the unabsorbed serum and all the tubes containing serum absorbed by Pneumococcus II contained a trace of scaly precipitate. Otherwise the results were unchanged.

specific substance until no further precipitate could be obtained after 2 hours at 37° and standing over night in the ice-box. The reaction of the purified gum with these sera and untreated Type II serum was then tested, giving the results shown in Table II.

4. Hydrolysis of the Specific Polysaccharide.

A. 41 gm. of preparation 51E were dissolved in water, treated with 85 cc. of concentrated sulfuric acid, diluted to 3 liters, and boiled for 4 hours, a preliminary experiment having shown a maximum reducing power after this time. The sulfuric acid was removed quantitatively with barium hydroxide, and to the filtrate, concentrated *in vacuo* to about 300 cc., basic lead acetate solution was

added in slight excess. The precipitate was suspended in water and treated with acetic acid in small amounts until only a small amount of yellow precipitate remained. The filtrate from this was again precipitated with an excess of basic lead acetate. The lead salt was decomposed with hydrogen sulfide and the solution concentrated repeatedly *in vacuo* to a syrup in order to eliminate acetic acid. The residue was taken up in hot water, boiled with acid-washed Norite, and concentrated to dryness, finally in a high vacuum. The yield of crude sugar acid was 5.5 gm. A determination of the reducing power by the Shaffer-Hartmann micromethod (8) gave a value of 44.6 per cent, calculated as glucose.

0.3329 gm., made up to 15 cc. with H_2O: α_D, 0.39°, $l = 2$. $[\alpha]_D = +8.8°$.

1 cc. of the same solution required 2.62 cc. 0.02 N NaOH for neutralization to phenolphthalein. Acid equivalent, 427. Calculated for $C_{12}H_{20}O_{12}$, 356.

The filtrates from the first and second precipitations of the lead salt were freed from lead, concentrated to small bulk, boiled with Norite, concentrated to a syrup, and, while still warm, were treated with about 2 volumes of glacial acetic acid and seeded with a few crystals of galactose. Crystallization took place rapidly, and the solid cake which formed over night in the ice-box was crushed, sucked off *in vacuo* on a Buchner funnel, and washed first with chilled 66 per cent acetic acid, then with the glacial acid, and finally with alcohol. The yield was 8.3 gm., with an initial $[\alpha]_D$ of +120.5°.

1 gm. of the crude sugar, oxidized with warm 1:1 nitric acid, began to deposit crystals within a few hours and ultimately yielded 0.46 gm. of mucic acid, melting at 214–215° with decomposition.

0.1009 gm. gave 0.1279 gm. CO_2 and 0.0458 gm. H_2O.

Calculated for $C_6H_{10}O_8$: C, 34.27 per cent; H, 4.80 per cent. Found: C, 34.57 per cent; H, 5.08 per cent.

6.8 gm. of the sugar itself were dissolved in water, boiled with Norite, and recrystallized as before, yielding 5.4 gm. of purified sugar, which from its analysis, rotation, and the isolation of mucic acid in good yield from the crude product, was chiefly galactose.

0.1007 gm. gave 0.1480 gm. CO_2, and 0.0620 gm. H_2O.

Calculated for $C_6H_{12}O_6$; C, 39.98 per cent; H, 6.72 per cent. Found: C 40.08 per cent; H, 6.89 per cent.

0.5513 gm., made up to 10 cc. with H_2O, gave an initial reading of 6.55° and a final value of 4.05°, $l = 1$. $[\alpha]_D$, initial, +118.8°; final, +73.5°.

The galactose isolated in this crop was not as pure as that recovered in later fractions (see below), but whether the impurity was the sugar acid still present in the mother-liquors, or some other sugar, has not been determined.

The filtrate from the initial crop of galactose was repeatedly diluted with water and concentrated *in vacuo* in order to remove acetic acid. It was then diluted to about 350 cc. and again treated with basic lead acetate solution, yielding a heavy

precipitate. This salt was worked up as was the first lead salt, and yielded 3.9 gm. of a crude sugar acid resembling the first product except in its rotation and somewhat lower reducing value, the latter being 39.2 per cent, calculated as glucose.

0.3064 gm., made up to 15 cc. with H_2O: α_D −0.17°, l = 2. $[\alpha]_D$ = −4.2°.

2 cc. of the same solution required 4.53 cc. 0.02 N NaOH for neutralization to phenol red. Acid equivalent, 450. Calculated for $C_{12}H_{20}O_{12}$, 356.

The filtrate from the above lead salt was freed from lead and acetic acid, taken up in a little water, neutralized with barium hydroxide, and again concentrated to a thick syrup. This was boiled with 3 successive 200 cc. portions of 90 per cent alcohol. The insoluble residue, purified over the lead salt, gave 2.0 gm. of a product which appeared to be a mixture of sugar and sugar acid, but was not further investigated. The alcoholic solutions after concentration to a syrup readily yielded 3.3 gm. of galactose, which melted at 161–163° after recrystallization.

0.5522 gm., made up to 10 cc. with H_2O, gave an initial reading of 7.52° and a final value, after addition of 0.5 cc. concentrated aqueous NH_3, of 4.11°, l = 1. $[\alpha]_D$, initial, +136.2°; final, +78.2°.

In Beilstein, 3rd edition, vol. i, p. 911, the rotations given for pure galactose are $[\alpha]_D^{20}$ +140°, 80.5°, respectively.

The mother liquors from the galactose contained but 1.7 gm. of sugar, calculated as glucose, and were not investigated.

(*B*).[2] 49 gm. of a product (active with Type II serum at a dilution of 1: 4,000,000) were hydrolyzed as in the previous instance. After removal of the sulfuric acid the concentrated solution was boiled with calcium carbonate and Norite, filtered, concentrated to small bulk, and fractionated with methyl alcohol as in the case of the calcium aldobionate derived from the Type III pneumococcus (3*b*). The partially purified salt thus obtained was further fractionated into three arbitrary portions with the aid of methyl alcohol and acetone.

Fractions	I	II	III	Theory
	per cent	*per cent*	*per cent*	*per cent*
Calcium	5.8	6.7	5.5	5.3
Reducing sugars (as glucose) (Schaffer-Hartmann)	44.8	44.3	34.4	48.0
Aldose (as glucose) (Willstätter-Schudel)	53.3	58.1	61.5	48.0

Fraction I[3] thus corresponds fairly closely to a calcium aldobionate, $(C_{12}H_{19}O_{12})_2Ca$, while the succeeding fractions show increasing contamination.

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[2] The experimental work in this section was carried out by one of us (M. H.) in the laboratories of the Mt. Sinai Hospital and the Presbyterian Hospital, New York. For the facilities offered by these institutions, and for the kindness of Dr. Forrest E. Kendall, of the Presbyterian Hospital, in carrying out the analyses, the writers wish to express their hearty thanks.

[3] A crystalline cinchonidine salt, and through this, the crystalline aldobionic acid have since been isolated, and will form the subject of a separate communication.

The analyses indicate that the chief impurity in Fraction II is possibly a salt of the type of calcium glucuronate, while Fraction III presumably contains galactose, especially as the mother-liquors from this deposited crystals of galactose on standing (melting point, 160–168°; yielded mucic acid, melting point, 206–207°, on oxidation).

DISCUSSION.

That a polysaccharide with specific properties should occur among the higher plants without any apparent relation to the life processes of micro-organisms, is additional evidence of the wide-spread occurrence of carbohydrates with immunologically specific properties. Speculation as to their function and chemical and immunological relationships, while enticing, must be postponed until more information is at hand. There is evidence which has been interpreted by Beijerinck (9) as pointing to the elaboration of gum arabic as a result of the activities of molds, and by Greig Smith (10) as showing the gum to be a product of the metabolism of certain bacteria. As neither of these workers has proved, however, that contamination of the gum with the appropriate organism did not take place after its formation the hypothesis that gum arabic originates through the activities of micro-organisms, though attractive from the standpoint of bacterial specificity, must be considered as unproved.

In fact, certain chemical data obtained in the present investigation argue against this point of view. On partial hydrolysis about one-half of the original material is recovered with its specific activity increased 100 to 150-fold, showing that the specifically reacting gum does not exist as such in the original gum arabic, but is formed from it, probably by removal of a pentose grouping in glucosidic union, since the specific fraction contains less than one-half the pentose of the gum arabic itself. The low reactivity of the original gum would then be accounted for on the basis of traces of the specific material formed on exposure, in the process of refining, or by enzyme action. It is not excluded however, that the specific gum owes its presence to the synthetic activity of the strong hydrochloric acid on the hydrolytic products of the original gum (11), although this would seem less probable.

Another finding of chemical interest is the large proportion of complex sugar acids. It has recently been shown (*3a* and *3b*) that the chief product of the hydrolysis of the soluble specific substance of

Type III pneumococcus is an aldobionic acid of which one component is glucose and the other glucuronic acid. A similar acid also forms one of the hydrolytic products of the soluble specific substance of the Type A Friedländer bacillus (12). At least one of the crude acid fractions from the hydrolysis of the specific gum arabic corresponds roughly to an aldobionic acid, while in the other the agreement is not so good. While both of these acid fractions may still be mixtures, their rough correspondence to important hydrolytic products of bacterial specific polysaccharides is of interest.

While the exact significance of these more or less complex sugar acids is still to be established, the finding of at least two of these acids as hydrolysis products of the specific gum arabic renders it evident that the specific gum, if actually a single substance in its present state of purity, is a more complex product than the bacterial specific polysaccharides hitherto investigated in detail. The recovery of the specific gum from its precipitate with Type II antipneumococcus serum with its reactivity for both Type II and Type III sera augmented (see experimental part) may be taken as evidence that it is a single constituent of the gum which precipitates both sera. This is in agreement with the relative complexity of the specific gum, since a substance containing the molecular groupings necessary for reaction with antibodies to both Type II and Type III pneumococci might be expected to yield more varied products on hydrolysis than one precipitating either serum alone. Whether any of these hydrolysis products of the specific gum arabic and the specific polysaccharides of Type II and Type III pneumococci are identical cannot be stated as yet. Thus far galactose has been found only in the first of the three, and this constitutes a marked difference.

From Table II it will be seen that Type II antipneumococcus serum which has been precipitated by the specific gum arabic still retains practically unimpaired its agglutinating power for the Type II pneumococcus and its precipitating power for the Type II soluble specific substance, whereas all of the antibodies are removed on absorption of Type II antiserum with Type II pneumococcus. This lack of reciprocal antibody absorption is suggestive of the relationship between Pneumococcus Type II and Type B Friedländer bacillus, in which the writers found (13) a certain chemical similarity between the specific polysaccharides of these bacteria and a corresponding immunological

relationship between the micro-organisms themselves. The serological and antigenic similarity of the otherwise unrelated bacteria was interpreted as an example of heterogenetic specificity; it is not unlikely that the reactions of specific gum arabic with Types II and III antipneumococcus sera may be accounted for in the same way.

SUMMARY.

1. By partial acid hydrolysis a specific carbohydrate may be isolated from gum arabic (gum acacia). This carbohydrate is comparable in its precipitating activity for Type II (and Type III) antipneumococcus serum with the bacterial soluble specific substances themselves.

2. On hydrolysis this fraction yields galactose and two or more complex sugar acids, one of which appears to be a disaccharide acid comparable with those isolated from the specific polysaccharides of the Type III pneumococcus and the Type A Friedländer bacillus.

3. The significance of these findings is discussed.

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BIOMETRY OF CALCIUM, INORGANIC PHOSPHORUS, CHOLESTEROL, AND LIPOID PHOSPHORUS IN THE BLOOD OF RABBITS.

III. Influence of Various Types of Light Environment.

By ALVIN R. HARNES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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In previous papers (1, 2) the calcium, inorganic phosphorus, cholesterol, and lipoid phosphorus found in the blood of rabbits as they were received from the dealer were compared with results obtained for animals living under laboratory conditions. The difference in mean values, in the variations that occurred, in the trends of the curves, and in the chemical relationship seemed to warrant the conclusion that all of these substances were affected by light. Since it had been found that calcium and inorganic phosphorus are affected by various types of light environment (3), it seemed desirable to extend the observations which had been made for the purpose of determining whether cholesterol and lipoid phosphorus were also affected by light and to study the action of a wider range of light conditions. The comparisons were made with complete exclusion of light, continuous exposure to light supplied by Cooper Hewitt lamps, exposure to diffuse filtered sunlight of variable intensity, and exclusion of light interrupted by brief periods of exposure to ultra-violet light. The results of these experiments will be reported in the present paper.

Materials and Method.

In the experiments presented, 3 animal rooms with similar environmental conditions other than those of lighting were employed. One group of 10 animals which may be designated as the open laboratory group, were kept in a well lighted (sunlight), well ventilated room with a southern exposure.

Two groups of 10 animals each were kept in a room from which all light was excluded. Other living conditions were the same as those of animals living in the open laboratory. During the course of the experiment, beginning on Feb. 1, 1928,

one group of animals was exposed for an hour each day to the unfiltered radiations of a quartz-mercury arc lamp (80 volts, 4.2 amperes) at a distance of 4.5 meters. These animals shall be designated as the ultra-violet group. The other animals which remained in the dark room throughout the experiment shall be designated as the dark group. Both of these groups of animals received some light from two other sources. For a brief period each day a 30 watt Mazda lamp was used in the dark room for cleaning cages, feeding, and making certain necessary observations. The animals were also exposed to diffuse filtered sunlight for a brief period once a week when they were brought into the laboratory for bleeding purposes.

The fourth group of animals which shall be designated as the light group was kept in a room similar to the others with sunlight excluded. In this room a *constant* source of light was furnished by 13 Cooper Hewitt low pressure mercury arcs, Type P, in crown glass arranged in 3 superimposed rows on an iron frame placed in the center of the room. The animals in individual cages were placed in racks on either side of and parallel with the mercury arcs at a distance of 1 meter. The average intensity of the light was calculated to be 2000 foot candles.

The spectrogram of this type of mercury arc in crown glass shows that the range of light rays is from 3022 to 5790 Ångström units with the majority falling in the 3650 and 5790 portion of the spectrum. The exposure to the Cooper Hewitt mercury arcs was begun on Jan. 20, 1928.

The doors of the cages were opened to permit free access of light but no depilatory measures were used for any group of animals, and at no time throughout the experiment could there be demonstrated evidence of dermatitis, conjunctivitis, or any other inflammatory process which might be attributed to the action of either ultra-violet or Cooper Hewitt radiation.

The temperature of the 3 rooms was satisfactorily maintained at 70° to 75°F. and the humidity of each varied with that of the outside atmosphere.

The animals selected for this experiment were all male rabbits of mixed grey and brown breeds from 6 to 8 months old. They were sexually mature but had not yet attained their full growth. All animals were caged separately and fed a uniform diet of hay, oats, and cabbage.

The data presented in this paper are derived from experiments, the procedures of which were described in detail in a preceding paper (1).

The determinations of calcium and inorganic phosphorus of blood serum, cholesterol and lipoid phosphorus of the whole blood were made on the same sample of blood. Determinations were made, beginning on Jan. 5, 1928, at weekly or biweekly intervals until May 31, 1928, resulting in 15 recorded observations for the light, dark, and open laboratory groups; 14 observations were made on the ultra-violet group.

In all text-figures and tables the lipoid phosphorus is calculated and presented as lecithin.

RESULTS.

The results of the observations made in this experiment are presented in the form of tabulated summaries, Tables I to VI, which are supplemented by a series of graphs, Text-figs. 1 to 14. The mean values contained in Tables I to VI inclusive have been smoothed by the formula $\frac{A + 2B + C}{4}$ and are presented in the graphs in terms of per cent variations from standard mean values, using for this purpose values obtained for animals living in the open laboratory throughout the year. These results were reported in a previous paper (2) and shall serve also as a basis of comparison for certain ratio values obtained in the present experiment. The average values for this group of animals were calculated to be for calcium 15.7, inorganic phosphorus 4.65 mg. per 100 cc. of blood serum, and for cholesterol 58.2, lecithin 118.4 mg. per 100 cc. of whole blood. Text-figs. 1 to 4 inclusive represent the per cent variation of the 4 blood constituents; Text-figs. 5 to 10 inclusive, the per cent variation of the 6 possible ratios of the same 4 blood constituents; and Text-figs. 11 to 14 inclusive, the distribution of individual values for calcium, inorganic phosphorus, cholesterol, and lecithin.

TABLE I.

Calcium Values for Consecutive Determinations.

Date	Open laboratory			Dark			Cooper Hewitt			Date	Ultra-violet		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation		Mean	Standard deviation	Coefficient of variation
1928	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*1928*	*mg. per 100 cc.*	*mg.*	*per cent*
Jan. 5	16.4 ±0.23	1.10	7.06	15.5 ±0.17	0.80	5.16	15.6 ±0.17	0.82	5.26				
" 19	15.2 ±0.14	0.67	4.45	15.3 ±0.20	0.95	6.22	15.2 ±0.11	0.55	3.62	Jan. 12	14.5 ±0.09	0.46	3.22
" 26	15.6 ±0.17	0.80	5.16	16.4 ±0.17	0.80	4.87	16.6 ±0.19	0.92	5.19	" 31	15.2 ±0.34	1.60	10.58
Feb. 2	15.3 ±0.10	0.48	3.15	15.3 ±0.10	0.48	3.16	15.7 ±0.15	0.72	4.58	Feb. 7	15.8 ±0.16	0.75	4.75
" 9	15.4 ±0.16	0.75	4.88	14.9 ±0.14	0.65	4.42	15.2 ±0.10	0.51	3.36	" 14	14.6 ±0.08	0.38	2.60
" 16	14.8 ±0.11	0.52	3.52	14.6 ±0.11	0.53	3.64	15.1 ±0.12	0.58	3.90	" 21	15.4 ±0.20	0.95	6.17
" 23	16.4 ±0.32	1.50	9.14	16.9 ±0.33	1.59	9.51	17.0 ±0.35	1.65	9.70	" 28	15.7 ±0.22	1.05	6.72
Mar. 1	15.8 ±0.04	0.19	12.34	16.3 ±0.14	0.67	4.11	16.1 ±0.11	0.54	3.35	Mar. 6	16.0 ±0.14	0.66	4.15
" 8	15.6 ±0.09	0.45	8.68	15.7 ±0.12	0.58	3.70	15.5 ±0.15	0.71	4.58	" 13	15.5 ±0.14	0.69	4.47
" 22	14.7 ±0.07	0.34	23.74	15.2 ±0.12	0.57	3.75	14.7 ±0.11	0.55	3.74	" 26	15.6 ±0.14	0.70	4.58
" 29	14.8 ±0.10	0.48	3.25	15.8 ±0.16	0.74	4.75	15.2 ±0.08	0.40	2.66	" 29	15.4 ±0.19	0.91	5.92
Apr. 12	14.8 ±0.13	0.62	4.15	15.0 ±0.12	0.57	3.82	14.9 ±0.16	0.61	5.35	Apr. 12	15.1 ±0.13	0.61	4.08
" 26	14.9 ±0.13	0.62	4.20	15.1 ±0.20	0.94	6.27	15.0 ±0.14	0.69	4.64	" 26	14.6 ±0.16	0.75	5.16
May 10	14.8 ±0.13	0.63	4.29	15.9 ±0.20	0.96	6.09	15.2 ±0.20	0.96	6.36	May 10	14.8 ±0.32	1.53	10.53
" 31	14.9 ±0.14	0.64	5.10	14.7 ±0.14	0.67	4.44	14.7 ±0.13	0.63	4.29	" 31	14.2 ±0.30	0.96	6.78

TABLE II.

Inorganic Phosphorus Values for Consecutive Determinations.

Date	Open laboratory			Dark			Cooper Hewitt			Date	Ultra-violet		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation		Mean	Standard deviation	Coefficient of variation
1928	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*1928*	*mg. per 100 cc.*	*mg.*	*per cent*
Jan. 5	5.43 ±0.10	0.48	8.87	5.64 ±0.11	0.53	9.55	5.50 ±0.11	0.55	10.10				
" 19	5.26 ±0.11	0.55	10.60	4.75 ±0.08	0.39	8.24	4.87 ±0.06	0.30	6.34	Jan. 12	6.10 ±0.16	0.78	12.85
" 26	5.40 ±0.06	0.32	6.01	4.53 ±0.10	0.48	10.63	5.27 ±0.08	0.37	7.13	" 31	5.17 ±0.10	0.49	9.61
Feb. 2	5.52 ±0.07	0.33	6.03	5.26 ±0.06	0.27	5.30	5.05 ±0.08	0.37	7.48	Feb. 7	4.99 ±0.11	0.52	10.46
" 9	5.58 ±0.07	0.34	6.24	4.73 ±0.05	0.26	5.68	5.01 ±0.07	0.34	6.96	" 14	4.84 ±0.13	0.62	12.83
" 16	5.97 ±0.09	0.46	7.75	5.13 ±0.09	0.45	7.91	5.44 ±0.07	0.33	6.22	" 21	4.43 ±0.03	0.17	3.90
" 23	5.73 ±0.11	0.51	9.95	5.18 ±0.16	0.79	15.36	5.26 ±0.07	0.36	6.93	" 28	5.40 ±0.15	0.71	13.14
Mar. 1	5.48 ±0.10	0.48	8.89	4.91 ±0.07	0.37	7.53	5.01 ±0.11	0.53	10.75	Mar. 6	5.96 ±0.13	0.64	11.31
" 8	5.15 ±0.05	0.24	4.83	4.73 ±0.08	0.41	8.74	5.01 ±0.06	0.27	5.55	" 13	4.94 ±0.09	0.43	8.76
" 22	4.78 ±0.07	0.34	7.18	4.71 ±0.08	0.40	8.58	4.65 ±0.06	0.32	6.99	" 26	5.14 ±0.08	0.39	7.65
" 29	5.05 ±0.10	0.48	9.52	4.67 ±0.06	0.30	6.61	5.18 ±0.06	0.30	5.92	" 29	6.58 ±0.20	0.94	14.29
Apr. 12	4.46 ±0.08	0.40	9.90	4.57 ±0.07	0.33	7.35	4.62 ±0.07	0.36	7.83	Apr. 12	4.99 ±0.08	0.40	8.18
" 26	4.47 ±0.07	0.34	7.77	4.57 ±0.07	0.34	7.48	4.77 ±0.10	0.47	9.84	" 26	5.02 ±0.09	0.42	8.54
May 10	4.99 ±0.10	0.51	10.26	4.92 ±0.09	0.43	8.81	4.92 ±0.09	0.43	8.92	May 10	5.53 ±0.08	0.41	7.55
" 31	4.80 ±0.08	0.36	8.10	4.27 ±0.08	0.40	8.61	5.07 ±0.08	0.36	6.96	" 31	4.87 ±0.09	0.43	8.72

TABLE III.

Cholesterol Values for Consecutive Determinations.

Date	Open laboratory			Dark			Cooper Hewitt			Date	Ultra-violet		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation		Mean	Standard deviation	Coefficient of variation
1928	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*1928*	*mg. per 100 cc.*	*mg.*	*per cent*
Jan. 5	65.6 ±1.23	5.76	8.78	63.4 ±1.18	5.54	8.74	61.8 ±0.75	3.54	5.74				
" 19	65.2 ±1.20	5.66	8.68	61.8 ±0.92	4.32	7.00	59.3 ±0.77	3.63	6.12	Jan. 12	73.3 ±1.35	6.36	8.68
" 26	61.9 ±1.39	6.56	10.59	57.0 ±1.18	5.56	9.75	56.1 ±0.82	3.88	6.82	" 31	57.5 ±1.23	5.76	10.03
Feb. 2	61.5 ±1.07	5.04	8.20	60.8 ±1.58	7.43	12.22	57.3 ±1.37	6.45	11.26	Feb. 7	60.3 ±0.84	3.71	6.33
" 9	61.2 ±1.26	5.92	9.67	58.3 ±1.25	5.89	10.11	55.2 ±0.73	3.45	6.25	" 14	65.8 ±0.94	4.41	6.70
" 16	61.6 ±1.28	6.04	9.81	58.3 ±1.10	5.18	8.88	62.1 ±0.91	4.29	5.90	" 21	71.2 ±2.18	10.24	14.38
" 23	60.0 ±1.77	8.30	13.83	58.5 ±0.83	3.93	6.72	55.6 ±1.31	6.14	11.04	" 28	66.0 ±1.63	7.64	11.58
Mar. 1	55.2 ±1.36	6.39	11.57	56.5 ±0.69	3.22	5.69	54.2 ±0.82	3.83	7.06	Mar. 6	57.0 ±0.66	3.10	5.96
" 8	58.7 ±1.47	6.89	11.73	65.8 ±1.54	7.20	10.94	58.0 ±0.80	3.74	6.45	" 13	60.9 ±1.20	5.63	9.24
" 22	59.5 ±1.46	6.84	11.49	65.7 ±1.32	6.20	9.43	64.4 ±1.11	5.19	8.05	" 26	59.1 ±1.15	5.40	9.14
" 29	63.9 ±1.91	8.96	14.02	57.7 ±1.00	4.70	8.14	59.7 ±1.05	4.90	8.21	" 29	73.4 ±1.10	5.15	7.02
Apr. 12	65.8 ±2.32	10.88	16.55	61.2 ±0.73	3.43	5.61	60.2 ±0.99	4.64	7.71	Apr. 12	69.6 ±0.96	4.51	6.47
" 26	71.2 ±2.60	12.20	17.13	64.0 ±1.81	8.48	13.25	66.9 ±1.95	9.15	13.67	" 26	68.3 ±1.43	6.72	9.84
May 10	64.7 ±1.59	7.46	11.53	67.8 ±1.66	7.76	11.45	61.7 ±1.56	7.30	11.80	May 10	80.3 ±1.72	8.03	10.01
" 31	66.2 ±1.22	5.69	8.72	59.9 ±0.92	3.84	6.38	83.8 ±1.36	6.38	10.49	" 31	67.7 ±1.30	5.82	8.76

TABLE IV.

Lecithin Values for Consecutive Determinations.

Date	Open laboratory			Dark			Cooper Hewitt			Date	Ultra-violet		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation		Mean	Standard deviation	Coefficient of variation
1928	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*1928*	*mg. per 100 cc.*	*mg.*	*per cent*
Jan. 5	129.8 ±2.97	13.11	10.10	137.9 ±2.38	11.17	8.10	143.2 ±0.15	7.07	4.93				
" 19	145.4 ±1.69	7.94	5.46	158.7 ±1.46	6.86	4.32	154.8 ±1.95	9.14	5.90	Jan. 12	125.1 ±1.63	7.65	6.12
" 26	112.1 ±2.16	10.17	9.07	110.7 ±2.60	12.19	11.01	119.2 ±2.09	9.80	8.22	" 31	109.6 ±1.10	5.19	4.74
Feb. 2	87.7 ±1.57	7.37	8.41	86.1 ±1.88	8.84	10.27	96.1 ±1.64	7.69	8.01	Feb. 7	124.6 ±2.91	13.65	10.95
" 9	145.3 ±2.21	10.39	7.15	155.4 ±2.37	11.15	7.18	151.5 ±3.90	18.29	12.07	" 14	141.9 ±1.90	8.92	6.29
" 16	116.8 ±2.75	12.90	11.04	108.9 ±4.21	19.75	18.13	121.2 ±3.46	16.23	13.39	" 21	144.9 ±3.51	16.48	13.73
" 23	138.4 ±3.18	14.89	10.75	129.1 ±6.34	29.75	23.04	115.5 ±3.87	18.15	15.71	" 28	95.7 ±2.24	10.53	11.00
Mar. 1	137.7 ±3.55	16.66	12.10	109.2 ±4.76	22.30	20.42	109.4 ±4.31	20.21	18.47	Mar. 6	96.1 ±2.43	11.39	11.85
" 8	77.8 ±4.02	18.85	24.22	75.1 ±3.69	17.31	23.05	73.6 ±2.37	11.10	15.08	" 13	134.5 ±3.16	14.81	11.01
" 22	147.2 ±2.34	10.95	7.43	162.5 ±4.30	20.15	12.40	159.0 ±4.61	21.60	13.52	" 26	96.7 ±2.13	9.97	10.32
" 29	139.1 ±2.71	12.70	9.13	122.3 ±3.09	14.50	11.85	130.4 ±3.84	18.01	13.81	" 29	111.0 ±1.86	8.72	7.85
Apr. 12	124.0 ±1.87	8.75	7.05	159.0 ±2.09	9.82	6.17	145.3 ±2.57	12.05	8.29	Apr. 12	126.9 ±4.39	20.62	16.34
" 26	129.2 ±3.48	16.33	12.63	134.4 ±2.65	12.45	9.26	126.6 ±4.73	22.19	17.53	" 26	128.4 ±3.27	15.35	11.95
May 10	129.5 ±4.51	21.15	16.33	135.4 ±3.73	17.48	12.91	135.0 ±2.55	11.95	8.85	May 10	123.6 ±3.30	15.45	12.50
" 31	119.9 ±1.66	8.31	7.68	126.1 ±1.44	6.21	5.37	117.1 ±1.67	8.93	7.52	" 31	135.4 ±1.81	10.11	9.46

TABLE V.

(*a*) *Coefficients of Correlation for Consecutive Group Means (Trend).*

Group	rP. Ca.	rP. Chol.	rP. Lec.	rCa. Chol.	rCa. Lec.	rChol. Lec.	
Open laboratory	+0.498 ±0.13	−0.566 ±0.12	−0.097 ±0.18	−0.328 ±0.15	−0.057 ±0.18	+0.117 ±0.17	N = 15
Dark room	+0.021 ±0.21	+0.065 ±0.18	−0.178 ±0.17	−0.180 ±0.17	−0.256 ±0.16	+0.147 ±0.17	N = 15
Light	+0.491 ±0.13	−0.146 ±0.17	−0.238 ±0.16	−0.627 ±0.10	−0.619 ±0.10	+0.776 ±0.07	N = 15
Ultra-violet	+0.027 ±0.21	+0.016 ±0.21	−0.722 ±0.08	−0.575 ±0.12	−0.625 ±0.10	+0.404 ±0.14	N = 14

(*b*) *Coefficients of Correlation for Individual Mean Values.*

Group	rP. Ca.	rP. Chol.	rP. Lec.	rCa. Chol.	rCa. Lec.	rChol. Lec.	
Open laboratory	−0.398 ±0.18	−0.110 ±0.21	−0.309 ±0.19	+0.034 ±0.22	+0.150 ±0.21	+0.064 ±0.22	N = 10
Dark room	−0.559 ±0.15	−0.008 ±0.22	−0.226 ±0.20	−0.028 ±0.22	+0.451 ±0.17	+0.061 ±0.22	N = 10
Light	−0.225 ±0.20	+0.331 ±0.19	−0.320 ±0.19	−0.607 ±0.13	−0.169 ±0.20	+0.403 ±0.18	N = 10
Ultra-violet	−0.258 ±0.20	+0.137 ±0.21	−0.683 ±0.11	+0.626 ±0.13	+0.646 ±0.12	+0.415 ±0.17	N = 10

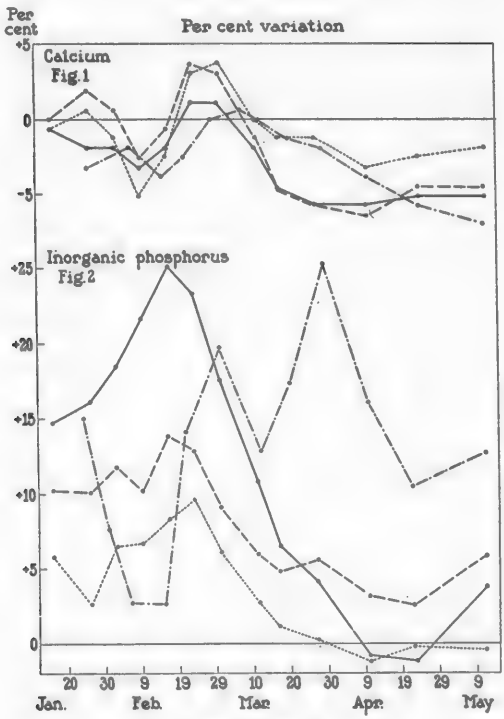
(*c*) *Coefficients of Correlation for Entire Series.*

Group	rP. Ca.	rP. Chol.	rP. Lec.	rCa. Chol.	rCa. Lec.	rChol. Lec.	
Open laboratory	+0.655 ±0.03	−0.241 ±0.05	−0.084 ±0.06	−0.069 ±0.06	−0.001 ±0.06	+0.027 ±0.06	N = 150
Dark room	−0.051 ±0.06	+0.045 ±0.06	−0.102 ±0.06	−0.075 ±0.06	−0.015 ±0.06	+0.169 ±0.05	N = 150
Light	+0.154 ±0.05	−0.052 ±0.06	−0.112 ±0.06	−0.398 ±0.04	−0.220 ±0.05	+0.113 ±0.05	N = 150
Ultra-violet	+0.031 ±0.05	+0.152 ±0.05	−0.306 ±0.04	+0.023 ±0.06	−0.115 ±0.06	+0.224 ±0.05	N = 140

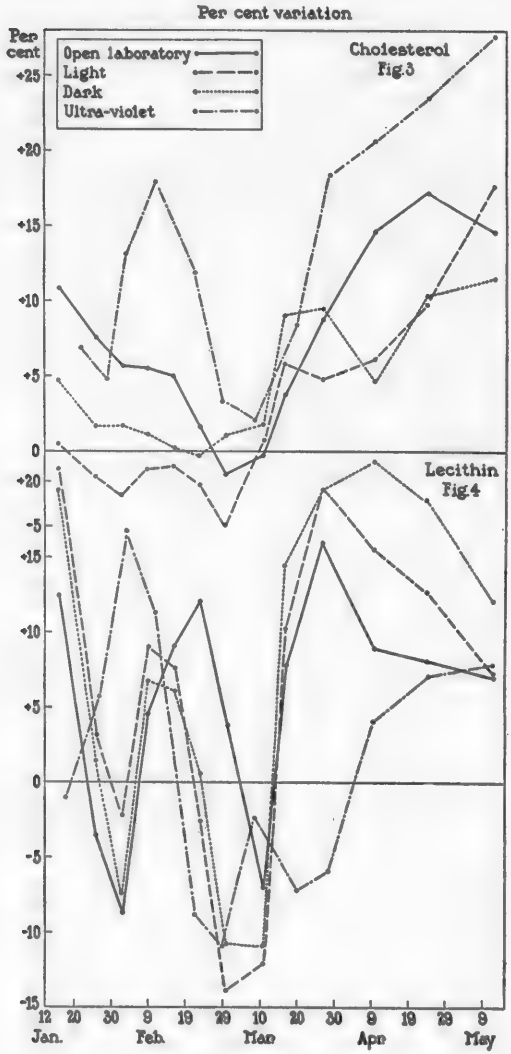
TABLE VI.

Mean Values for Each Group.

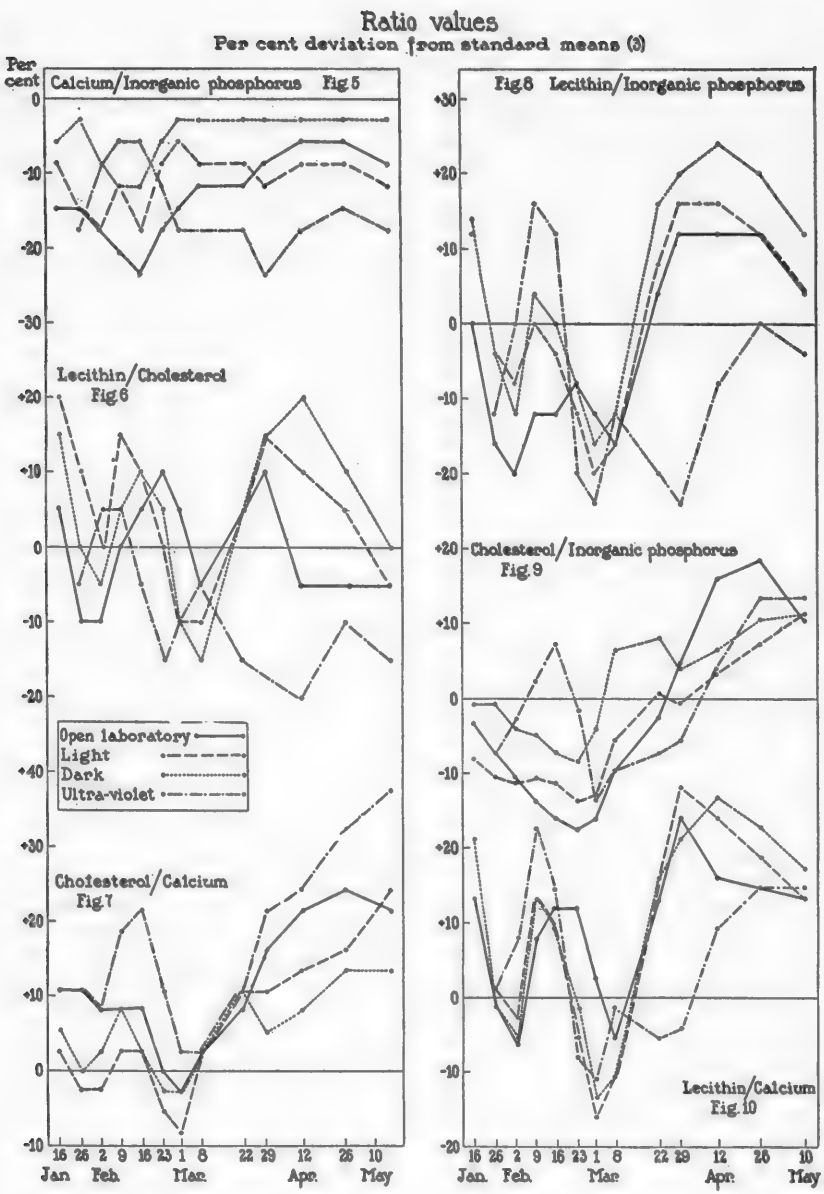
Group	Calcium	Phosphorus	Cholesterol	Lecithin	N
Open laboratory.....	15.3 ±0.05	5.21 ±0.01	62.7 ±0.45	124.0 ±1.33	150
Dark room.....	15.6 ±0.05	4.83 ±0.02	60.9 ±0.38	126.0 ±1.65	150
Light.....	15.5 ±0.05	5.02 ±0.02	59.8 ±0.42	125.0 ±1.35	150
Ultra-violet.....	15.3 ±0.05	5.26 ±0.04	65.9 ±0.51	120.0 ±1.22	140



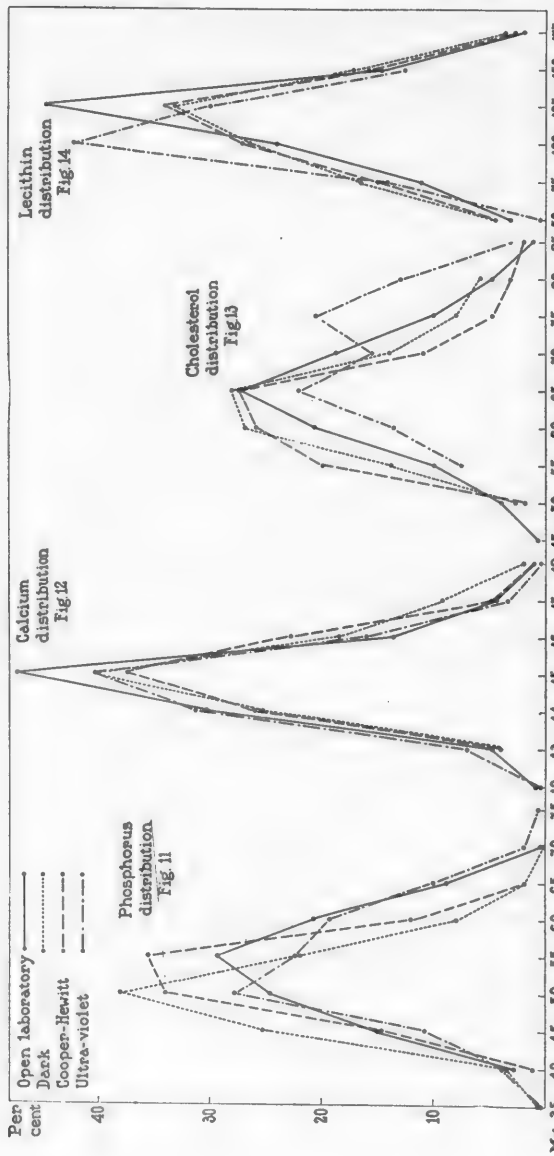
FIGS. 1 AND 2.


Per cent variation
Per cent
Open laboratory
Light
Dark
Ultra-violet
Cholesterol
Fig.3
Lecithin
Fig.4
+25
+20
+15
+10
+5
0
+20
-5
+15
+10
+5
0
-5
-10
-15
12 20 30 9 19 29 10 20 30 9 19 29 8
Jan. Feb. Mar. Apr. May

FIGS. 3 AND 4.


Ratio values
Per cent deviation from standard means (3)
Per cent
Calcium/Inorganic phosphorus Fig.5
Lecithin/Cholesterol Fig.6
Cholesterol/Calcium Fig.7
Open laboratory
Light
Dark
Ultra-violet
Fig.8 Lecithin/Inorganic phosphorus
Cholesterol/Inorganic phosphorus Fig.9
Lecithin/Calcium Fig.10
16 26 2 9 16 23 1 8 22 29 12 26 10
Jan Feb Mar Apr May

FIGS. 5 TO 10.


Per cent
Open laboratory
Dark
Cooper-Hewitt
Ultra-violet
Phosphorus distribution Fig. 11
Calcium distribution Fig. 12
Cholesterol distribution Fig. 13
Lecithin distribution Fig. 14
Mg

FIGS. 11 TO 14.

DISCUSSION AND CONCLUSIONS.

Variation.

Calcium.—The element calcium (Text-fig. 1) proved to be the most stable and least variable of the 4 blood constituents. This was true for each of the 4 groups of animals.

The trend of calcium in the blood serum of animals living in the open laboratory showed a gradual decrease from the beginning of the experiment on Jan. 5 until about Feb. 9. Throughout the next 3 weeks the calcium in this group of animals increased in amount until on Feb. 29 it reached its highest level. From this high value the calcium decreased until about Mar. 20 at which time it had reached a more or less stable level which was maintained during the remaining 2 months of the experiment.

The animals living in the dark and those exposed to Cooper Hewitt light showed similar trends for the first 3 months of the experiment but exhibited a degree of variation as to the general levels of calcium metabolism. These groups of animals showed first an increase in calcium and then a rather rapid decline, reaching their minimum level at the same time as those animals living in the open laboratory, the dark group slightly lower, and the light slightly higher than those living in the open laboratory. From the low values of Feb. 9, the calcium of both groups of animals increased in amount, reaching their maximum on Feb. 29. Throughout the initial period of the experiment, the animals in the dark room maintained a lower level of calcium than those exposed to the Cooper Hewitt light. This difference, however, was reversed, and from Feb. 29 throughout the remaining time of the experiment, the animals living in the dark room maintained a higher level of calcium than any of the others, while the light group followed closely the trend of the animals living in the open laboratory.

The animals exposed to ultra-violet rays showed first, a slight increase of calcium and then a decrease, this decrease occurring at a time when the trend of calcium for the 3 other groups was on the increase. The ultra-violet group reached its highest level of calcium on Mar. 6, this occurring at a time when the trend of all other groups was downward. From this time until the end of the experiment, the calcium for the ultra-violet group continued to decrease.

During the last month of the experiment the 4 groups of animals exhibited 4 distinct levels for the calcium content of blood serum. The animals living in the dark room had the highest amount of calcium, those exposed to Cooper Hewitt light ranking next with a smaller amount, the third group, those animals living in the open laboratory with a slightly lower amount of calcium than the light group, while the least amount of calcium during this last 4 weeks' period was found to be in the animals exposed to the ultra-violet light.

Inorganic Phosphorus.—The inorganic phosphorus of the blood serum (Text-fig. 2) ranked next to calcium with a higher degree of variation. This fact held true for each of the 4 groups of animals. The greatest variation occurred in the group of animals living in the open laboratory while the animals in the dark room showed the least variation. The most abrupt changes in inorganic phosphorus were noted to occur in the blood serum of those animals that were exposed to ultra-violet light.

Analyzing the curves for the individual groups, it will be noted that with the animals living in the open laboratory there occurred a gradual increase in inorganic phosphorus which reached its maximum amount on Feb. 16. This gradual increase was not unlike that which occurred in the group of animals previously reported (2) and it will also be noted that this maximum occurred at a time when the previously reported group gave its minimum values, *i.e.*, at about 6 weeks from the beginning of the experiment. From this high value, the inorganic phosphorus in the serum of animals living in the open laboratory exhibited a gradual and uninterrupted decrease, reaching its lowest level on Apr. 12. The last 3 determinations gave only slight increases in inorganic phosphorus over this minimum value.

The animals exposed to Cooper Hewitt light showed the same initial rise in inorganic phosphorus as the animals living in the open laboratory. It will be noted, however, that at the beginning of the experiment the light group maintained a decidedly lower level than those in the open laboratory, but the maximum values for the 2 groups of animals occurred at about the same time. From this high point on Feb. 16, the inorganic phosphorus showed a gradual decrease which continued until Apr. 12, the last 3 determinations giving increasing amounts over this minimum value. At the beginning of the experi-

ment the level of inorganic phosphorus for the light group was far below that of the open laboratory group, but this order was reversed on about Mar. 20, and from this time until the end of the experiment the light animals maintained a higher level of inorganic phosphorus than those living in the open laboratory.

Except for a slight decrease at the beginning of the experiment, the trend of inorganic phosphorus in the serum of animals living in the dark room was the same as that found in the light and open laboratory groups. With the dark group the maximum value, which occurred Feb. 23, came 1 week later than the maximum of either the light or open laboratory groups. From this high amount, the inorganic phosphorus in the blood serum of the dark animals gradually decreased in amounts until about Apr. 9, when the minimum for the entire experiment was obtained. It will be noted that the general level of inorganic phosphorus maintained by the dark group was below that of the light, and except for one determination, that of May 19, was far below the level maintained by the animals living in the open laboratory. The animals living in the dark room showed the least variation.

The inorganic phosphorus of the ultra-violet group exhibited trends and variations in a direction opposite to that of the 3 other groups of animals. At the beginning of the experiment this group of animals gave the highest value but during the succeeding 4 weeks the inorganic phosphorus decreased, reaching its minimum on Feb. 21. It may be noted that this continued decrease occurred over a period of time when the 3 other groups of animals were exhibiting trends of increasing amounts. From this minimum, the ultra-violet group showed a gradual increase in inorganic phosphorus and except for the 2 determinations on Mar. 10 and 20, reached their maximum on Mar. 30. This high value for the ultra-violet group was reached at a time when the 3 other groups had more or less stabilized at their respective minimum levels. From this maximum, the inorganic phosphorus showed decreasing amounts for the 2 next determinations and was followed by increasing amounts for the 2 last examinations.

During the last 10 weeks of the experiment, the 4 groups of animals maintained distinct levels of inorganic phosphorus, these levels being independent and except for one value found in the dark group, showed no overlapping. The highest level of inorganic phosphorus was main-

tained during the last 10 week period by the ultra-violet group followed in order by the light group and animals living in the open laboratory, with the dark group at the lowest level.

Cholesterol.—The variability exhibited by the cholesterol (Text-fig. 3) in the blood of the 4 groups of animals was practically the same as that of inorganic phosphorus.

The group of animals living in the open laboratory showed a gradual decrease in cholesterol from the beginning of the experiment, reaching their minimum on Feb. 29. This low value occurred at a time when animals living out of doors, *i.e.*, just received from the dealer, were maintaining their maximum level of cholesterol for the entire year. From this minimum the cholesterol in the blood of the open laboratory group rapidly increased, the maximum value occurring on Apr. 19. The 2 last determinations showed only a slight decrease from this maximum value.

At the beginning of the experiment the group of animals exposed to the Cooper Hewitt light showed the least amount of cholesterol, but during the last 10 weeks gave values which were only slightly less than those for the group living in the open laboratory. The light group at the beginning of the experiment showed a gradual decrease in cholesterol which was followed by a slight increase during February, and a second decrease which reached its minimum on Feb. 29. Throughout this entire period the light group showed the least cholesterol of any of the 4 groups. During the remainder of the experiment the trend of cholesterol for this group of animals was upward, except for the one determination on Mar. 29, and a level below the open laboratory group was maintained until the last examination was made.

The animals living in the dark room maintained a level of cholesterol which in general was between the values shown by the light and open laboratory groups, but during the last 4 weeks was just below the animals exposed to Cooper Hewitt light. From the beginning of the experiment the cholesterol decreased until a minimum was reached on Feb. 23. This was followed by a gradual increase until Mar. 9 at which time a marked decrease in cholesterol was noted. The following determinations, however, gave values which represented the maximum for this group for the entire time of the experiment.

The group of animals exposed to the ultra-violet light gave 2 values

for cholesterol at the beginning of the experiment which were slightly below that obtained for the group in the open laboratory. The ultra-violet group then showed a rapid increase in cholesterol, reaching a maximum for this period on about Feb. 14. This increase occurred at a time when the cholesterol of all other groups was decreasing. The trend of cholesterol following this high point was downward until Mar. 10 when it reached its minimum. From this minimum the cholesterol exhibited a very rapid increase which continued throughout the remainder of the experiment. It will be noted that except for the 2 values at the beginning of the experiment and the one obtained on Mar. 20, the general level of cholesterol for the ultra-violet animals was markedly higher than that for any of the 3 other groups of animals. For the last 6 weeks of the experiment, in the order of decreasing levels, the ultra-violet group ranked first; the group living in the open laboratory came next with practically 10 per cent less cholesterol; the light and dark groups followed with the light group slightly higher in cholesterol than the dark.

Lecithin.—The abruptness and degree of variation proved to be greater for lecithin (Text-fig. 4) than for any of the 3 other blood constituents. This fact held true for each of the 4 groups of animals. The animals living in the open laboratory, the dark and light groups, listed in the order of increasing values for lecithin at the beginning of the experiment, showed a decreasing trend which reached a minimum on Feb. 2. From this point, the 3 groups exhibited a rather abrupt increase in lecithin, the group living in the open laboratory reaching its maximum about Mar. 1 which was 10 days later than the maximum for the light or dark groups. During the following 4 weeks, there occurred another decrease in lecithin in each of these 3 groups of animals which terminated with minimum values for the 3 groups on Mar. 10.

During the last 8 weeks of the experiment, the 3 groups of animals showed a rapid increase in lecithin, the dark group showing the greatest amount throughout this period; the light group next with slightly less; and the open laboratory group with the lowest level for the 3 groups.

The animals exposed to the ultra-violet light maintained the highest level of lecithin during the first part of the experiment, but during the

last 8 weeks they were the lowest of the 4 groups. Beginning with lecithin far below the 3 other groups, the ultra-violet animals showed a rapid increase which reached its maximum about Feb. 21. This occurred at a time when the 3 other groups were exhibiting decreasing trends. From this high point, the lecithin decreased and reached a minimum about the same time as the light and dark groups, this being the lowest point attained by the ultra-violet group throughout the experiment. A slight increase in lecithin was found at the next examination, but this was followed by 2 determinations of decreasing values. It will be noted that this decrease occurred at a time when the 3 other groups exhibited their greatest per cent increase. From this low level the lecithin in the ultra-violet group gradually increased and at the last determination was slightly higher than for the group living in the open laboratory or the group exposed to Cooper Hewitt light. The general level of lecithin maintained by the ultra-violet animals throughout the last 8 weeks of the experiment, however, was markedly lower than that of the 3 other groups.

Considering the calcium, inorganic phosphorus, cholesterol, and lecithin throughout the course of the experiment in all groups of animals, it will be noted that the curves representing the trend of these 4 blood constituents may in general be divided into two periods. The first 8 weeks of the experiment showed the greatest degree of irregularity in trend and frequency of variation. This period, as suggested by Brown (3) may be called the period of adjustment or accommodation to changed environment. In this experiment the period of accommodation was about 2 weeks longer than that required by the group of animals previously reported (2) but this difference in time may be attributed to the difference of environmental conditions. The second period, comprising the last 8 weeks of the experiment, was characterized by relative stability. During this last 8 weeks, each group of animals exhibited and maintained more or less distinct levels for each of the 4 blood constituents which can only be explained on the basis of differences in light environment, since all other factors for the 4 groups of animals were, as far as possible, the same.

The mean values for calcium, inorganic phosphorus, cholesterol, and lecithin maintained by these 4 groups of animals over the entire period of the experiment are presented in Table VI. These values

show differences of the same general order as those indicated in the text-figures.

Ratios.

The 6 possible combinations of calcium, inorganic phosphorus, cholesterol, and lecithin in the form of ratios are presented in Text-figs. 5 to 10 inclusive. In order that each group might serve as a control for the others all values were reduced to terms of a percentage deviation from a standard mean as explained above.

Each of the 6 ratio text-figures is divisible into two sections as in the case of the curves showing the variations in the amounts of the 4 substances. The first period of some 8 weeks represents the period of accommodation to the changed environment. It will be noted here, as in the previous curves, that all ratios during the first 8 weeks exhibited wide degrees of variation and rather abrupt changes, and that it was not until the last 8 weeks of the experiment that any degree of stability was maintained by al groups of animals. Since the true effect of the conditions studied is shown best by the results obtained during the last 8 weeks of the experiment, the discussion will be limited to this period.

The most consistent and the highest calcium-inorganic phosphorus ratio (Text-fig. 5) was maintained by the animals living in the dark room. While both calcium and inorganic phosphorus showed a certain degree of variation during this period of the experiment, the ratio between the 2 substances was practically constant. A change in relative position took place between the light and open laboratory animals during the last 5 weeks' period. At the beginning, the light group exhibited a higher ratio of calcium-inorganic phosphorus, but fell slightly below the open laboratory groups after Mar. 22. This change in ratio value was due primarily to the drop in inorganic phosphorus of the animals in the open laboratory below that maintained by the light group.

The lowest calcium-inorganic phosphorus ratio during this period of the experiment was maintained by the animals exposed to ultraviolet light. This low ratio was due to both a decrease in calcium and an increase in inorganic phosphorus. The order into which these 4 groups of animals aligned themselves with respect to the calcium-

inorganic phosphorus ratio was first, the animals living in the dark with the highest ratio; second, the open laboratory group; third, the light group; and fourth, the ultra-violet group with the smallest ratio value. It will be noted that all values were lower than those for the standard group (3). This was due mainly to a difference in calcium values.

Stability in the lecithin-cholesterol ratios (Text-fig. 6) for all groups of animals did not occur until some 10 days after the calcium-inorganic phosphorus ratios had been established at more or less definite levels. The delay in lecithin-cholesterol stabilization was due chiefly to the variation which occurred in the cholesterol content of the blood during this period.

The highest lecithin-cholesterol ratio was maintained by the animals living in the dark room. At the beginning of the last 8 weeks' period, the ratio maintained by the light group coincided with that of the dark, but during the final 5 weeks, there was a separation of the 2 groups, the ratio of the light falling below that of the dark group.

The animals living in the open laboratory showed the least variation in the lecithin-cholesterol ratio. With the beginning of the last 8 weeks' period, this group gave ratio values below both the dark and light groups. The final ratios were somewhat lower than that maintained by either the light or dark groups.

The animals exposed to the ultra-violet light maintained the lowest lecithin-cholesterol ratio. This low value was maintained throughout the last 8 weeks. Listing the 4 groups of animals in the order of the relative magnitude of the levels maintained, the animals in the dark room came just with the highest lecithin-cholesterol ratio; the light group second; the open laboratory group came third; and the ultra-violet animals fourth with the smallest ratio values. The low position of the ultra-violet group was the result of the high cholesterol (Text-fig. 3) and the comparatively low lecithin values (Text-fig. 4) exhibited throughout this last 8 weeks' period.

The cholesterol-calcium ratios (Text-fig. 7) for the 4 groups of animals showed levels which were just the reverse of the lecithin-cholesterol ratios. The ultra-violet groups maintained the highest cholesterol-calcium ratio throughout the last 8 weeks; the open laboratory group, except for the last value, came next with a slightly lower

ratio; then the light group; and the animals in the dark with the lowest value for this period of the experiment.

The ratio of lecithin to inorganic phosphorus (Text-fig. 8) was highest in animals living in the dark room. The light and open laboratory groups came next with a slight decrease in ratio values, and it will be noted that at the time of the 2 last determinations these groups gave values which coincided.

The ratio of lecithin to inorganic phosphorus maintained by the ultra-violet group was markedly below that found in any other group of animals. This low ratio was due mainly to a high inorganic phosphorus, but the lecithin was also lower than in the other animals.

The cholesterol-inorganic phosphorus ratio (Text-fig. 9) showed marked delay in stabilization with more overlapping of the trends than any of the other ratios. This condition may be ascribed to the wide variation in cholesterol content of the whole blood than to the inorganic phosphorus which exhibited a much greater degree of stability and maintained a definite separation of levels for each of the 4 groups of animals.

The animals living in the open laboratory gave the highest cholesterol-inorganic phosphorus ratio except for the last determination at which time these animals were the lowest of the 4 groups. The ultra-violet group, which at the beginning of this period gave values below those for all other animals, came next with a slightly lower ratio; the final value, however, was slightly above that obtained for the 3 other groups.

The animals in the dark room exhibited a greater stability with respect to their cholesterol-inorganic phosphorus ratio than the other groups of animals. While at the beginning of this period the ratio values of the dark group were well above those of other animals, the subsequent increase was less than that of the open laboratory and ultra-violet groups, both of which rose to a higher level than the dark group. At the last determination, the ratio for the animals in the dark coincided with that found for the light group. The animals exposed to Cooper Hewitt light maintained a cholesterol-inorganic phosphorus ratio below that of the dark group throughout the experiment, except as pointed out, at the time of the last examination when these 2 ratios were the same.

The results of the 6 ratio curves (Text-figs. 5 to 10) with respect to relative position of each group of animals during the last 8 weeks of the experiment are presented on a comparative basis in the following table, the figure 1 representing the highest and 4 the lowest ratio value.

Group	Ca./P.	Lec./Chol.	Chol./Ca.	Lec./P.	Chol./P.	Lec./Ca.
Dark room	1	1	4	1	2	1
Cooper Hewitt	3	2	2	2	3	2
Open laboratory	2	3	3	3	1	3
Ultra-violet	4	4	1	4	4	4

These results show definitely that the group of animals in the dark room and those exposed to ultra-violet light occupied diametrical positions with respect to their ratio levels. Except for 2 ratios, the animals in the open laboratory maintained values next to that of the ultra-violet group, while the light group gave results nearer those of animals in the dark.

Distribution.

The distributions of all values obtained for each of the 4 substances studied are presented according to groups in Text-figs. 11 to 14 inclusive. These curves are similar in form to those previously reported (1) except for the one curve representing the distribution of cholesterol for the ultra-violet group of animals. The irregularity in this curve is probably due to the extreme high values obtained for these animals during the last 6 weeks of the experiment. The differences in position of the other curves correspond to the results presented in Text-figs. 1 to 4.

Correlation.

The coefficients of correlation for the 6 possible combinations of calcium, inorganic phosphorus, cholesterol, and lecithin are presented in Table VI. Section (a) gives the results obtained by calculating the correlation coefficients using mean values for each group and, therefore, presents the relationship with respect to trend. Except for

*r*P.Ca. which gives a positive correlation for the 4 groups of animals, the remaining 5 correlations were the same with respect to sign as those presented in a previous report (1). The highest degree of correlated trend was found in the *r*Chol. Lec. of animals exposed to the Cooper Hewitt light. In the *r*P. Lec. the ultra-violet group gave the highest degree of correlated trend.

In section (b) of the same table the correlation coefficients are calculated on the basis of the mean values maintained by each individual animal throughout the experiment. The signs of the *r*P.Ca. in this section are negative, while in the previous section they are positive. This is due to the fact that while individual animals maintained negative relationships in their corresponding sigma values, the general trend of the mean values for calcium and inorganic phosphorus tend to parallel each other in degree and direction of variation.

The correlation coefficients in section (c), calculated on the basis of a comparison of single determinations with the means for the entire series, give values which in the majority of cases are the same as section (a).

Throughout the 3 sections it will be noted that the *r*Chol.Lec. were all positive in sign, the magnitude of the coefficients varying somewhat with respect to the different groups and method of calculation. These results correspond with those reported in a previous paper (1) and seem to indicate that the 2 lipoid substances, cholesterol and lecithin, parallel each other in variation and trend.

The relationship existing between the inorganic phosphorus of the blood serum and the lecithin of the whole blood is of especial interest. Reviewing the results so far obtained with respect to these two substances, it will be noted that lecithin in all cases has proved to be the most variable constituent and has shown the most abrupt and most frequent change of trend. The coefficient of variation and standard deviation of inorganic phosphorus emphasize the stability of this substance when compared to the lipoid fraction. It seems probable, therefore, that there is a reciprocal relationship existing between inorganic phosphorus and lecithin and also the lipoid fraction may serve as a reservoir or source of supply for the inorganic form.

The results show that animals when brought into the laboratory pass through a period of adjustment or accommodation which in this

experiment covered a period of about 8 weeks. The mechanism by which these effects are induced is unknown.

The results obtained from this experiment are interpreted as furnishing evidence that exposure to the ultra-violet light for a comparatively brief period daily results in a definite change in levels of calcium, inorganic phosphorus of the blood serum and cholesterol and lecithin of the whole blood. Animals excluded from the effects of all light, as far as is practicable, maintained levels of the 4 blood constituents diametrical to the group of animals exposed to ultra-violet rays. The animals living in the open laboratory and those exposed to the Cooper Hewitt light exhibit levels of the 4 blood constituents which are somewhat similar in position, but both groups maintained values just between those of the dark and ultra-violet groups of animals.

SUMMARY.

Experiments are reported in which it was shown that the calcium, inorganic phosphorus, cholesterol, and lecithin in the blood of normal rabbits were influenced by 4 types of light environment. The results of the experiments seem to warrant the following conclusions:

1. Animals exposed to the ultra-violet light for a brief period each day give results which are diametrical to those obtained for animals living in total darkness.

2. The results obtained for animals exposed to the Cooper Hewitt light and for those living in the open laboratory are somewhat similar but occupy a position between those of the dark and ultra-violet groups.

3. Animals do not develop immediately the characteristic effects of a particular environmental condition, but pass through a period of accommodation which varies somewhat with different environmental conditions.

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REACTIONS OF RABBITS TO NON-HEMOLYTIC STREPTOCOCCI.

II. Skin Reactions in Intravenously Immunized Animals.

By HOMER F. SWIFT, M.D., and C. L. DERICK, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

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In previous papers it has been shown (1, 2) that rabbits inoculated in practically any manner, except intravenously, with sufficiently large doses of certain non-hemolytic streptococci developed a condition of tissue hypersensitiveness. This was made evident by the occurrence of secondary reactions at the site of the primary inoculation, by the presence of corneal sensitivity after a certain period, by much larger reactions following intracutaneous re-inoculation than occur in normal animals inoculated with similar doses of culture; and finally by death of many of the animals following intravenous inoculation with amounts of culture well tolerated by normal rabbits. It was also shown that if the primary inoculation of the animals had been by the intravenous route, using amounts of culture and time intervals comparable with those employed in the hypersensitized (*i.e.*, intracutaneously inoculated) rabbits, these intravenously inoculated animals responded with none of these reactions of hypersensitiveness. From such observations one might have concluded that they reacted to re-inoculation in the same manner as normal animals; but on closer study it was found that their reactivity differed from that of either normal or hypersensitive animals. The object of this communication is to present the macroscopic evidence that these intravenously inoculated rabbits react differently than do normals to intracutaneous inoculation.

EXPERIMENTAL.

The methods employed were comparatively simple: animals were inoculated intravenously with varying amounts of centrifugate of 18 to 24 hour blood broth cultures of non-hemolytic streptococci, and

after varying periods were inoculated into the previously depilated skin with decreasing amounts of similar cultures of homologous micro-organisms. The test doses were usually 10^{-1} cc., 10^{-2} cc., 10^{-3} cc., and 10^{-4} cc., the first in 0.1 cc. volume, the other three in 0.05 cc. volumes, as it was found that with these amounts distinct differences in reaction of the various animals were made quite clear. At the time of the skin testing a group of normal control animals was inoculated in a similar manner; hence the strength of the culture used in any given experiment was determined.

Experiment 1.—Two rabbits, Q515 and Q516, were inoculated intravenously on Feb. 5 and Feb. 15, 1926, with the centrifugate of 5 cc. of culture of Streptococcus V92/0/11. On Feb. 19 they, together with 3 normal controls, Q531, Q532, Q533,

TABLE I.

Average Size of Lesions following Intracutaneous Inoculation of Rabbits Previously Inoculated Intravenously, Compared with Controls.

	No.	Size of inoculum		
		10^{-1} cc.	10^{-2} cc.	10^{-3} cc.
		*mm.**	*mm.*	*mm.*
Intravenously inoculated	2	33	15	Negative
Normals	3	39	20	9

* Indicates average sum of 2 longest diameters of the lesions.

were inoculated intracutaneously with 10^{-1} cc., 10^{-2} cc., and 10^{-3} cc. of the homologous strain. The lesions were measured and described daily. 2 days later a striking difference in the reactions of the 2 groups of animals was evident: At the site of the 10^{-1} cc. inoculation of Rabbits Q515 and Q516 there were red, hard, shotty lesions; at the site of the 10^{-2} cc. lesions there were flat, barely palpable, pink, maculopapules, and where 10^{-3} cc. had been injected there was no macroscopic evidence of injury. The controls at this time showed larger and softer lesions resulting from inoculation of 10^{-1} cc. and 10^{-2} cc. doses and with one exception also distinct soft papules at the site of the 10^{-3} cc. inoculation.

Because all of the rabbits received intravenous inoculations 7 days later the subsequent development of the lesions was probably altered, hence the main differentiation between the 2 groups appears in the physical characteristics of the reactions and in the average size of comparable lesions in the 2 sets of animals, as shown in Table I.

The results of a different time interval between the immunizing and testing doses are shown in Experiment 2.

Experiment 2.—Each of 4 animals, Q380, Q381, Q385, and Q386, received intravenously the centrifugate of 10 cc. of blood broth culture, V92/0/10. 20 days later the reactivity of their skins, together with that of 4 controls, was tested with doses of 10^{-1} cc., 10^{-2} cc., and 10^{-3} cc. of homologous culture. The results are shown in Table II.

TABLE II.

Comparison of Skin Reactivity of Rabbits Intravenously Immunized 20 Days Previously with That of Normal Animals.

	Rabbit No.	Sum of diameter of lesions			Secondary reaction	Retested after 10 days; sum of diameter of lesions		
		Size of inoculum				Size of inoculum		
		10^{-1} cc.	10^{-2} cc.	10^{-3} cc.		10^{-1} cc.	10^{-2} cc.	10^{-3} cc.
		mm.	mm.	mm.		mm.	mm.	mm.
Immunized intravenously	Q380	55	22	16	0	44	23	16
	Q381	30	30	16	0	37	26	22
	Q385	46	26	24	0	52	24	16
	Q386	32	23	16	0	33	29	17
Average.....		41	25	18		41	25	18
Normal	Q415	36	22	16	+	76	30	24
	Q416	37	20	0	+	46	27	18
	Q417	49	25	27	0	47	25	16
	Q418	50	19	0	0	46	27	21
Average.....		43	21	11		54	27	20

It is at once obvious that the differences in the size of primary reactions between the intravenously inoculated group and normals was not so marked as in Experiment 1. Neither was the nodular character of the lesions of the immunized group so marked as in the first experiment. Nevertheless, 2 of the 4 normal rabbits showed secondary reactions at the sites of the 10^{-1} cc. and 10^{-2} cc. inoculations, while none of the immunized animals showed secondary reactions.

It thus appears that differences in the interval between immunizing inoculation and skin testing had a distinct influence on the character of the reaction following intracutaneous inoculation. When this period was short, as in Experiment 1, the differences between the

immune and normal animals were greater than in Experiment 2, where an interval of 20 days had elapsed. It is probable that the relatively slight immunity that followed the single intravenous injection of 10 cc. of streptococci was passing off by this time. Still it was sufficient to prevent the development of secondary reactions, and an accompanying general hyperergy; for the immunized group retested 10 days later showed practically the same sized lesions as when first tested, while the controls similarly retested showed distinctly larger lesions.

The effect of more prolonged continuous intravenous inoculation is brought out better in Experiment 3.

Experiment 3.—A group of 7 animals, R651, R652, R653, R654, R655, R657, and R658, were selected for immunization with culture of Streptococcus V110A. Five of them were inoculated intravenously as follows: 1st day 1 cc., 3rd day 1 cc., 5th day 2 cc., 7th day 4 cc., 12th day 5 cc. Two received only 1 cc. each on the 11th and 14th days respectively. On the 16th day all were tested with intracutaneous inoculations of 10^{-1} cc. and 10^{-2} cc. of blood broth culture of homologous streptococci; 7 normal rabbits were tested with similar doses. On the 18th day the first 5 rabbits of the first group each received the centrifugate of 4 cc. of culture intravenously and the other 2 cc. On the 27th day all received 4 cc.; thus the immunization was continued during the period in which secondary reactions might have been expected to appear.

The results of these intracutaneous inoculations are shown graphically in Charts 1 and 2. In Chart 1 are given the curves formed from daily measurements of the sum of the 2 longest diameters of the lesions, while in Chart 2 the volumes of the lesions resulting from the 10^{-2} cc. inoculations are indicated.[1] A distinct difference is immediately evident. Only 3 out of the 7 rabbits inoculated intravenously showed secondary reactions, but 2 of these had received only 2 small inoculations before the skin testing; the third showed only a slight and late secondary reaction. Six out of the 7 normal animals, on the other hand, developed secondary reactions, which in most instances usually appeared about the 8th to the 10th day; none of the immune animals, on the other hand, showed secondary reactions before the 14th day. The primary reactions of the 2 groups of animals also showed similar differences in consistency and size to those previously noted. These differences in size are brought out more strikingly by comparing their

[1] The method of calculating these volumes is given in a previous paper (2).

volumes as shown in Chart 2, where not only the greater initial size of the primary lesions, but also that of the secondary reactions in the

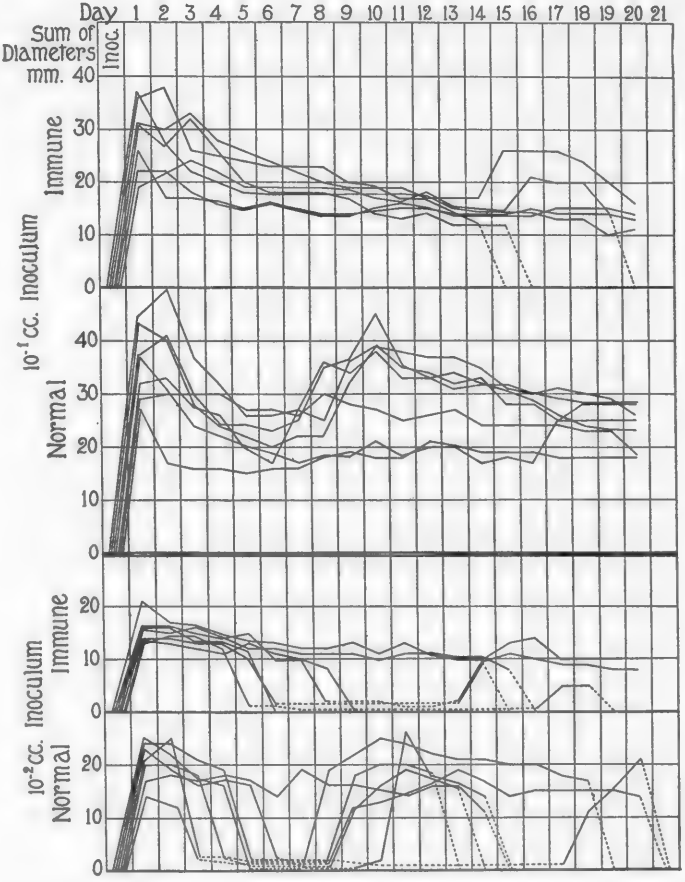

CHART 1. Comparison of sizes of lesions of immune and normal rabbits following intracutaneous inoculation with Streptococcus V110A.

normal animals is made evident. The immunized animals showed only relatively small increases in their lesions at the time of their secondary reactions, while the normal animals showed very marked in-

creases. This experiment indicates how necessary it is not only to measure the diameters, but also to pay careful attention to the thickness of these lesions. The nodular character of the primary reactions in the immune group compared with softer lesions in the normal animals was observed here as in previous experiments.

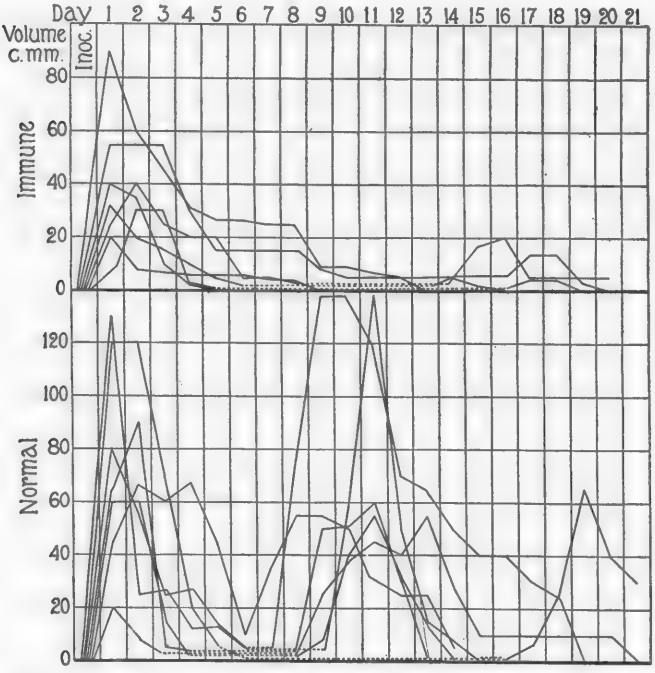

CHART 2. Comparison of volumes of lesions of immune and normal rabbits following intracutaneous inoculation with 10^{-2} cc. of blood broth culture of Streptococcus V110A.

In view of the fact that such differences in the tissue response could be demonstrated when animals were previously inoculated intravenously with living organisms, it was decided to investigate whether or not a similar alteration in responsive capacity could be detected if a bacterial fraction were used as the immunizing agent. It had been previously demonstrated by Lancefield (3) that the nucleoprotein ex-

tract of green streptococci was the only fraction with a definite antigenic capacity. She, therefore, prepared a fairly large quantity of nucleoprotein of Streptococcus V92 with which a group of rabbits was immunized intravenously, as indicated in Experiment 4.

Experiment 4.—Eight rabbits were injected intravenously with a normal saline solution of green streptococcus nucleoprotein as follows: 1st, 2nd and 3rd days, 10 mg. each day; 9th, 10th, 11th and 12th days, 20 mg. each day; 17th, 18th, 19th and 20th days, 30 mg. each day. On the 26th day each rabbit received intracutaneous inoculations with the centrifugate of blood broth culture of Streptococcus V92 in the following amounts, 5 cc., 5 cc., 10^{-1} cc. and 10^{-2} cc.; 4 normal controls were similarly inoculated. The curves showing the reactions of 4 of the immunized animals compared with the 4 controls are shown in Charts 3 and 4. Only the curves of the 10^{-1} cc. and 10^{-2} cc. lesions are given because the intensity of the response to 5 cc. of culture was so marked in all rabbits that differences in the 2 groups were not made so evident as with the smaller inocula.

While differences in the diameters of the primary lesions in the 2 groups of animals were not very marked, it became apparent, as the evolution of the lesions was followed, that there was a distinct difference in the response of the animals. All of the normal controls showed well marked secondary reactions in all of their lesions, while only 3 of the 8 immunized animals developed secondary reactions in the smaller lesions; and in 2 instances these were slight and delayed. The striking difference in the character of these secondary reactions is brought out in Chart 4, where the volume of the 10^{-2} cc. lesions of 4 of the immunized rabbits is compared with similar lesions in the 4 controls.

While the average volume of the primary lesions was 69 c.mm. in the immunes compared with 94 c.mm. in the controls, that of the secondary reactions was 25 c.mm. in those developing these reactions compared with 230 c.mm. in the controls. It is evident, therefore, that immunization with nucleoprotein over a period of approximately 4 weeks had altered the rabbits' type of response towards intracutaneous inoculation in the direction of a diminished intensity of reaction. It must be realized that the amount of nucleoprotein used represented large bacterial growths, and that probably a similar period of immunization with a corresponding amount of vaccine would have been more efficacious. The main point to be gathered from this ex-

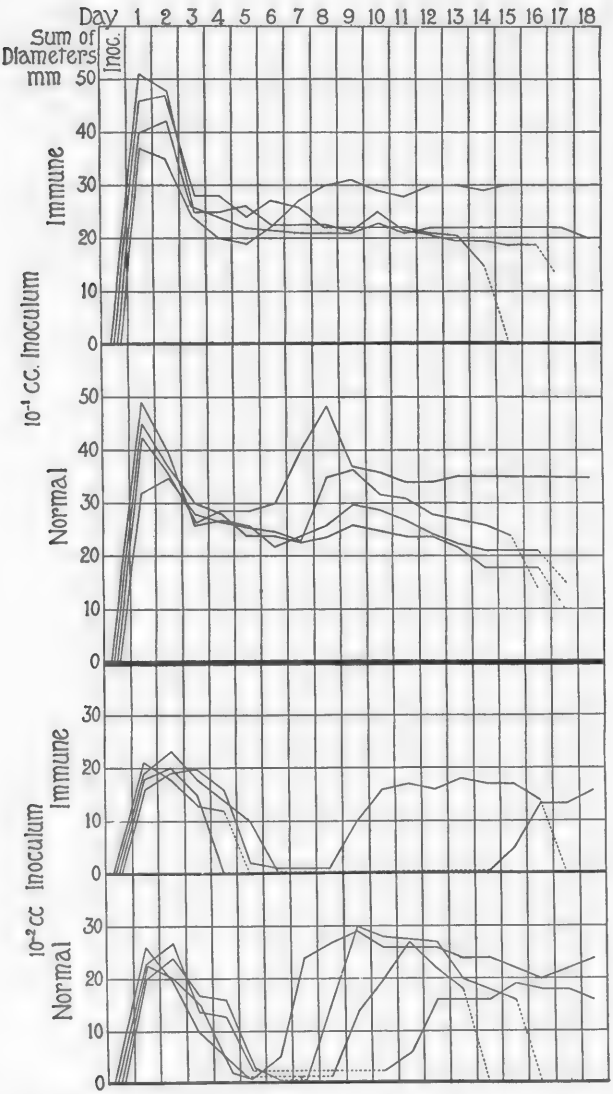

CHART 3. Comparison of sizes of lesions of rabbits immunized with nucleoprotein of green streptococci and normal rabbits following intracutaneous inoculation with Streptococcus V92.

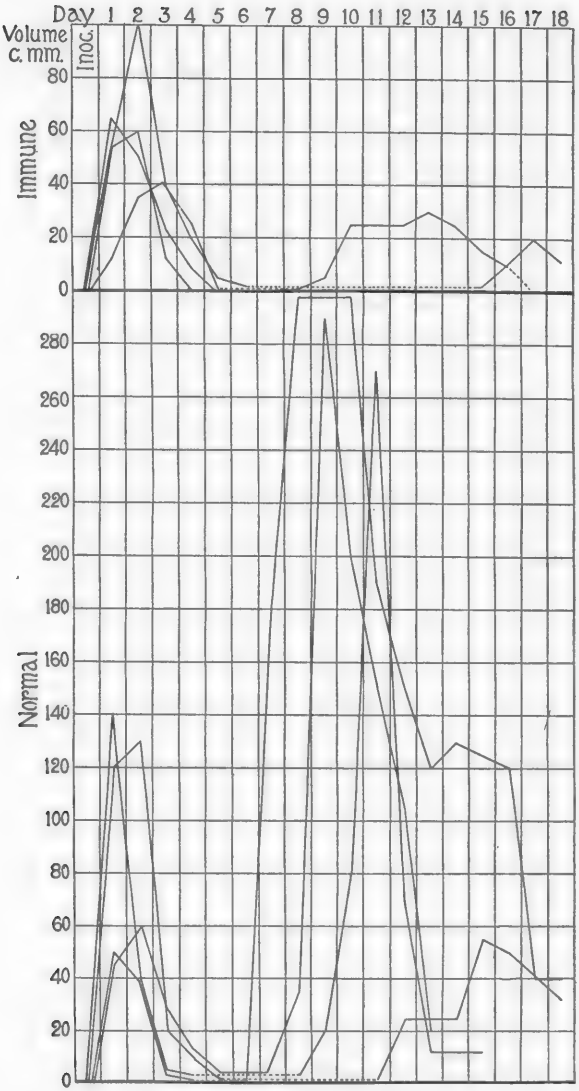

CHART 4. Comparison of volumes of lesions of rabbits immunized with nucleoprotein of green streptococci and normal rabbits following intracutaneous inoculation with 10^{-2} cc. of blood broth culture of Streptococcus V92.

periment was that the nucleoprotein fraction carried the immunizing substance.

As a final experiment the effect of prolonged intravenous immunization was studied as shown in Experiment 5.

Experiment 5.—Twelve rabbits were inoculated intravenously over a period of 9 weeks. In the beginning 0.5 cc. of whole blood broth culture of Streptococcus V110A was employed. This was gradually increased to 2 cc. after 10 days; but the rabbits began to lose weight so rapidly that immunization was discontinued for a week, then recommenced with 1 cc. of heat killed culture. This, in turn, was gradually increased until 5 cc. doses were given; subsequently immunization with living culture was resumed without depressing the rabbits. During the succeeding period of 4 weeks 9 inoculations were given by gradually increasing the dose from 1 cc. to 4 cc. of living blood broth culture. At the end of this period the sera of all the rabbits showed a precipitin titer of 1:4000 to 1:8000 when tested against nucleoprotein prepared from Streptococcus V110A. The agglutinin titer of the sera could not be determined because of spontaneous agglutination of the cultures.

After this course of 9 weeks' immunization the skin reactivity of all the rabbits together with that of 4 normal controls was tested with Streptococcus V110A/0/5 in 6 doses of from 10^{-1} cc. to 10^{-6} cc. None of the animals showed reactions at the sites of the 10^{-4} cc., 10^{-5} cc., and 10^{-6} cc. inoculations. The maximum and average sizes of the lesions in the 3 larger lesions in all of the animals are shown in Table III; also the volumes of the 10^{-2} cc. and 10^{-3} cc. lesions are given.

While quantitative variations in the response of the different animals occurred, there was almost a uniform tendency for the immunized animals to show smaller lesions than the controls. The ratio of the averages in the immune group compared with the normals at the sites of the different inocula was 1^{-1} cc. 2:3; 10^{-2} cc. 1:2; 10^{-3} cc. 2:3. When volumes of lesions are compared, just as in previous experiments, the differences are even more striking. In the 10^{-2} cc. lesions the average ratio was 1:3, and in the 10^{-3} cc. lesions 4:9. The daily notes of the consistency of the lesions also brought out striking differences. The immune animals had very hard shotty lesions, particularly at the sites of 10^{-2} cc. inoculations, while the normal animals showed some edema with only moderately infiltrated lesions from the 1st to the 3rd days. These differences were so striking that the immune animals could be readily differentiated from the controls by simple comparison of the size and character of the corresponding lesions.

None of the immune animals showed secondary reactions in any of

their lesions, while 3 out of the 4 controls developed secondary reactions. It may be objected that the differences in the size of the 2 groups tend to invalidate the comparisons, but all of 4 other normal rabbits inoculated with a corresponding culture 2 days later developed primary lesions and secondary reactions similar to these 4 controls; so it may

TABLE III.

Comparison of Skin Reactivity of Rabbits Immunized Intravenously Over a Long Period with Normal Controls.

	Rabbit No.	Sum of diameters of lesions			Volume of lesions	
		Size of inoculum			Size of inoculum	
		10^{-1} cc.	10^{-2} cc.	10^{-3} cc.	10^{-2} cc.	10^{-3} cc.
		mm.	*mm.*	*mm.*	*c.mm.*	*c.mm.*
Immunized intravenously	1119	40	17	12	98	15
	1120	35	17	v. and p.*	90	v. and p.*
	1122	42	21	13	117	17
	1123	34	19	12	108	6
	1124	24	16	9	50	2
	1125	40	25	15	165	22
	1131	38	16	12	10	14
	1135	47	18	10	54	2
	1136	53	28	20	188	60
	1138	38	20	14	106	20
	1139	33	20	15	100	12
	1140	42	28	15	157	22
Average.....		39	20	12	110	16
Normal	1253	70	39	20	517	55
	1254	52	27	15	150	10
	1255	53	34	18	315	48
	1256	78	38	17	423	45
Average.....		63	37	17	334	37

* Non-measurable, visible and palpable.

be safely concluded that these controls represented the average response of normal animals at this period. This experiment, therefore, indicates that prolonged intravenous immunization causes a group of animals to react to subsequent intracutaneous inoculation in a more uniform manner than does short immunization such as was carried out with living streptococci in the earlier experiments, or with streptococcus nucleoprotein as in Experiment 4.

DISCUSSION.

It is common knowledge that previous inoculation alters the reaction of an animal towards an infectious agent; in fact upon this knowledge are based our efforts towards the prevention and cure of infectious diseases where the various techniques of immunology are employed. In certain conditions the outstanding defensive agents are easily demonstrable in the body fluids in the form of antitoxins, opsonins or similar antibodies. In other diseases, where the invasive microorganisms have relatively low virulence, or elaborate comparatively little exotoxin, the defensive mechanism seems to reside in the cells rather than in the humors of the body. But in most infections both humoral and cellular factors apparently play a rôle; and in proportion as one or the other predominates, so, in part at least, may differences in the focal manifestations of infection and resistance be explained.

Following Pirquet's (4) observations and introduction of the term allergy, studies of the local manifestations of reinfection have assumed a constantly increasing importance, until at the present day an enormous literature has grown up about this word. It seems to be quite generally accepted that the phenomenon of allergy represents a very intense local effort on the part of the animal to limit the activity of infectious agents or foreign substances to the site where they gain entrance into the animal's body. It is also obvious that local reactions are quite different following the introduction of such agents as vaccinia, tubercle bacilli, egg albumin or primin into suitably allergized animals or men. Re-inoculation with vaccine virus results in smaller local injury than occurs at the time of first inoculation, while reinjection with egg albumin leads to more marked local reaction, which increases in intensity with increase of circulating antibodies (5). Re-inoculation with tubercle bacilli, on the other hand, is followed by destructive localized tissue reaction, the Koch phenomenon, without any corresponding increase of antibodies in the blood serum. Repeated treatment of the skin with primin, a nitrogen free ether extract of primrose results in a type of allergy made manifest by an eczematous inflammation of the skin (6). In these four examples of allergy the differences in local response may be explained in part by differences in the nature of the antigen or inciting agent. Following the introduction of tubercle bacilli into the body tubercles are formed

with an accompanying general state of tuberculin allergy regardless of the presence of demonstrable circulating antibodies. Egg albumin, on the other hand, may be introduced intravenously into an animal without producing gross focal injury; nevertheless with the development of antibodies a state of allergy ensues. It is therefore apparent, as pointed out by Zinsser (7), Doerr (8), Coca (9), and others, that the allergy induced by bacteria and by coagulable proteins is different. In practically all instances in which a tuberculin-like allergy is induced this follows some focal tissue reaction resulting from injury by the respective micro-organism.

But, in so far as we are aware, no comparison has previously been made of the type of tissue reaction which follows the introduction of bacteria into the body so that in the one instance gross lesions are produced and in the other no macroscopic lesions are formed. One difficulty attending such experimentation has been due to the fact that most bacteria, previously used in the study of allergy, induced focal lesions regardless of the route employed for inoculation. But with non-hemolytic streptococci, such as we have employed, these conditions could be more easily controlled. Strains with which it was possible to induce a condition of tuberculin-like allergy when they were injected into the tissues, could also be used to decrease the animal's tissue reactivity when they were injected intravenously in suitable doses. Ordinarily streptococci of this type quickly disappear from the blood stream without producing gross lesions; but in some instances, especially when large doses are employed, the animals develop endocarditis or arthritis. In one rabbit of Experiment 3, in which an arthritis of the wrist had followed the early intravenous inoculation, the skin test inoculation was followed by lesions of the hypersensitive rather than of the immune type. This isolated example taken in conjunction with other experiments previously reported (2) supports the viewpoint that focal lesions are probably necessary for the development of hypersensitiveness of infection.

Another point worthy of note is that intravenously immunized rabbits when inoculated intracutaneously with suitable doses show lesions of a different character than do either normal or hypersensitive animals, lesions that have little if any edema and are hard and firm after 24 to 48 hours. In fact these lesions show much less change in size

after 2 days than do the lesions in other types of animals. The larger inocula often are followed in 4 or 5 days by globular sac-like areas persisting for weeks, and smaller inocula produce small hard persistent nodules. In other words, the entire and complete reaction seems to take place much more quickly in the immune than in other states of allergy. The microscopic comparison of the different types of lesions will be reported in a later communication.

Finally, for purposes of completeness it should be noted that certain rabbits give reactions following intracutaneous inoculation which differ from any of those previously described by us. These reactions are usually less marked than those of normal animals, are soft, have very little color, fade rapidly, and do not show secondary reactions. They occur in rabbits which appear sick, either due to an overwhelming infection from streptococci, or from any other cause. These animals are usually emaciated and have a skin which is wrinkled, gray and lacking in tone. We have designated these reactions "cachectic," and after a little experience have learned to recognize them within 2 or 3 days following intracutaneous inoculation. Obviously the inclusion of such animals in any group is to be avoided for they vitiate statistical comparisons.

Within recent years it has become evident to many observers that different states of resistance towards a given bacterium could be indicated by differences in cutaneous reactivity. For example, a patient showing well marked positive reaction to intracutaneous injections of tuberculin may lose this skin reactivity during an attack of measles or other infectious disease, or if he becomes cachectic from a neoplasm or even from an overwhelming tuberculous infection. This has been designated as negative anergy, and corresponds with what we call cachectic reaction. It has also been observed that an animal may show decreasing skin reactivity to a certain fixed dose of a testing bacterial extract while it is becoming more resistant to a general infection with the same bacterium (10). This state has been designated as positive anergy, and probably corresponds with our designation, immune reaction. It seems probable, then, that the term allergy will have to be qualified in some manner, if it is to be continued as a means of expressing the idea of increased resistance accompanied by over-reaction of the tissues. For this reason we have at times used the term *hyperergy* which has been employed by a number of German

pathologists (11, 12) to indicate a hyper-reactivity of the tissues, and the terms immune or cachectic to indicate gross decreases in reactivity according to the manner in which this decreased tissue reactivity is induced.

SUMMARY.

Rabbits immunized intravenously with living culture or nucleoproteins of non-hemolytic streptococci react to subsequent intracutaneous inoculations with homologous streptococci with smaller and harder lesions than are shown by normal animals similarly inoculated; and they do not develop the general manifestations of hypersensitiveness such as are shown by animals previously inoculated into the tissues with the same cultures.

A rabbit may react to intracutaneous inoculation with non-hemolytic streptococci in one of four ways, depending on whether it is normal, hypersensitive, immune or cachectic. Most normal animals show a secondary reaction about 10 days after inoculation with suitable strains of non-hemolytic streptococci; hypersensitive, allergic, or hyperergic animals show much larger lesions than do normals with the corresponding doses of the same streptococci, and practically never show secondary reactions; immune animals show smaller and harder early lesions and usually do not have secondary reactions if they are fairly well immunized. Cachectic animals show very soft and rapidly fading primary reactions and no secondary reactions.

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CONTENTS

BROWN, HERMAN, and RAMSDELL, SUSAN GRIFFITH. Blood calcium distribution in anaphylaxis in the guinea pig 705

COULTER, CALVIN B., and ISAACS, MOSES L. Oxidation-reduction equilibria in biological systems. II. Potentials of aerobic cultures of *B. typhosus* 711

FURTH, J., and LANDSTEINER, K. Studies on the precipitable substances of bacilli of the Salmonella group 727

MCMASTER, PHILIP D., and DRURY, D. R. The production of partial liver insufficiency in rabbits 745

DRURY, D. R. Total surgical removal of the liver in rabbits 759

DRURY, D. R., and MCMASTER, PHILIP D. The relation of the liver to fat metabolism. I. Effect of liver lack on fat combustion and the respiratory quotient 765

MUDD, STUART, LUCKÉ, BALDUIN, MCCUTCHEON, MORTON, and STRUMIA, MAX. On the mechanism of opsonin and bacteriotropin action. I. Correlation between changes in bacterial surface properties and in phagocytosis caused by sera of animals under immunization 779

LUCKÉ, BALDUIN, MCCUTCHEON, MORTON, STRUMIA, MAX, and MUDD, STUART. On the mechanism of opsonin and bacteriotropin action. II. Correlation between changes in bacterial surface properties and in phagocytosis caused by normal and immune sera 797

MCCUTCHEON, MORTON, STRUMIA, MAX, MUDD, STUART, MUDD, EMILY B. H., and LUCKÉ, BALDUIN. On the mechanism of opsonin and bacteriotropin action. III. The development and effect of the antibodies found in experimental tuberculosis of rabbits 815

RATNER, BRET, and GRUEHL, HELEN LEE. Transmission of respiratory anaphylaxis (asthma) from mother to offspring 833

HEIDELBERGER, MICHAEL, AVERY, OSWALD T., and GOEBEL, WALTHER F. A "soluble specific substance" derived from gum arabic 847

HARNES, ALVIN R. Biometry of calcium, inorganic phosphorus, cholesterol, and lipoid phosphorus in the blood of rabbits. III. Influence of various types of light environment 859

SWIFT, HOMER F., and DERICK, C. L. Reactions of rabbits to non-hemolytic streptococci. II. Skin reactions in intravenously immunized animals 883

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NOGUCHI, HIDEYO. The etiology of trachoma. Plates 1–31.

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